METHOD DEVELOPMENT FOR THE DETERMINATION OF HYDROXYLAMINE IN RUBBER SAMPLES

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(APPLIED ANALYTICAL AND INORGANIC CHEMISTRY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2016

Thesis
entitled

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METHOD DEVELOPMENT FOR THE DETERMINATION OF HYDROXYLAMINE IN RUBBER SAMPLES

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science
(Applied Analytical and Inorganic Chemistry)
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ACKNOWLEDGEMENTS

I would like to express my sincere thanks to a number of people who gave me the invaluable help and the comfortable encouragement to make this thesis through. Firstly, this thesis could not be completed without the guidance and support from my advisor, Dr. Tinnakorn Tiensing who gave me knowledge, shared experiences, and taught me a life. I express my truly gratitude to Assoc. Prof. Atitaya Siripinyanond and Asst. Prof. Rattikan Chantiwas, my co-advisor, for all kind assistance throughout my project.

I am appreciative to Michelin Siam Company for all caring of this project and the financial research support and also thankful to the scholarship from an Institutional Strengthening Program and a Teaching Assistance funded by the Faculty of Science, Mahidol University.

I also express my thank to all members in Applied Analytical and Inorganic Chemistry Program (AAICP) for their great friendship, support, and morale and special thank to my all teacher for providing me a lot of knowledge.

Finally, my endless gratitude goes to my dear and lovely family who always beside me and give me eternal love, warm happiness, and invaluable support.

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METHOD DEVELOPMENT FOR THE DETERMINATION OF HYDROXYLAMINE IN RUBBER SAMPLES

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ABSTRACT

Hydroxylamine (NH_2OH) is an additive compound in natural rubber (NR) used to prevent the 'storage hardening' of NR. The amount of NH_2OH in NR needed to be measured before use because of the effect to viscosity of NR.

This research developed three analytical methods for the determination of NH_2OH in NR samples, i.e., high performance liquid chromatography (HPLC) with cation-exchange column, HPLC with reversed-phase column, and colorimetry using Fe(II)-*o*-phenanthroline complex. NR samples used in this work were obtained from Michelin company and Rubber Technology Research Centre (RTEC). NH_2OH was extracted from rubber by two extraction methods, i.e., solid-liquid extraction (SLE) and reflux.

The developed and optimized processes of the analysis were carried out by using NH_2OH standard. First, HPLC method with cation-exchange column; Synchrom S300 (250 mm × 4.6 mm); flowed with the mobile phase of ammonium acetate buffer:acetonitrile showed poor repeatability response and unacceptable recovery value. It was resulted from the side reaction of NH_2OH with acetonitrile in the mobile phase. Second, the method of HPLC with reversed-phase column was carried out by forming acetone oxime derivatives between NH_2OH and acetone. The separation was achieved on LiChroCART® (250 mm × 4 mm, RP-18 (5 µm)) column using 10:90 (%v/v) methanol:DI water as the mobile phase and the UV detector was set at 230 nm. The method gave linearity in the range of 0.5-50.0 mg/L with good precision (< 1.4%RSD). Detection limit (DL) was 0.16 mg/L. Third, the colorimetric method was an indirect analysis of NH_2OH by reducing Fe(III) to Fe(II) and then forming red color complex of Fe(II)-*o*-phenanthroline. The absorbance of the complex was measured by spectrophotometer at 510 nm. The method showed linearity in the range of 0.05-2 mg/L and DL was 0.003 mg/L. Finally, these developed methods were applied for determining quantity of NH_2OH in the rubber samples.

For Michelin samples, the recovery was obtained at lower than 2.9% when it was extracted by SLE and lower than 13.5% by reflux. For RTEC samples, it showed higher recovery value than Michelin samples. The recoveries were in the range of 41.2-82.8% by SLE and 31.3-56.0% by reflux.

KEY WORDS: HYDROXYLAMINE / NATURAL RUBBER / HPLC / Fe(II)-*o*-PHENANTHROLINE

90 pages

การพัฒนาวิธีการวิเคราะห์สำหรับการตรวจวัดไฮดรอกซีลามีนในตัวอย่างยาง
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บทคัดย่อ

ไฮดรอกซีลามีนเป็นสารเติมแต่งในยางธรรมชาติถูกใช้เพื่อป้องกันการแข็งตัวของยางระหว่างการเก็บรักษา ปริมาณไฮดรอกซีลามีนในยางธรรมชาติจำเป็นต้องถูกตรวจวัดก่อนการใช้งาน เพราะผลกระทบต่อความหนืดของยางธรรมชาติ

งานวิจัยนี้ได้ทำการพัฒนาวิธีวิเคราะห์สำหรับการตรวจวัดไฮดรอกซีลามีนในตัวอย่างยางธรรมชาติทั้งหมด 3 วิธี ได้แก่ เทคนิคเทคนิคไฮเพอร์ฟอแมนซ์ลิควิดโครมาโตกราฟี (HPLC) ร่วมกับการใช้คอลัมน์แลกเปลี่ยนประจุบวก, เทคนิค HPLC ร่วมกับคอลัมน์รีเวิร์สเฟส และเทคนิคการวัดเชิงแสงโดยใช้การตรวจวัดสารประกอบเชิงซ้อนของเหล็ก(II)-ออร์โท-ฟีแนนโทรลีน ตัวอย่างยางธรรมชาติที่ใช้ในงานนี้ได้รับมาจากบริษัทมิชลิน และจากศูนย์วิจัยเทคโนโลยียาง ไฮดรอกซีลามีนถูกสกัดออกจากยางโดยใช้วิธีสกัด 2 วิธีได้แก่ การสกัดของแข็ง-ของเหลว และการสกัดร้อนแบบไหลย้อนกลับ

กระบวนการของการพัฒนาและการหาค่าที่ดีที่สุดของการวิเคราะห์ถูกทำโดยใช้สารมาตรฐานไฮดรอกซีลามีน วิธีแรกคือเทคนิค HPLC กับคอลัมน์แลกเปลี่ยนประจุบวก, Synchrom S300 (250 mm × 4.6 mm), โดยใช้เฟสเคลื่อนที่เป็นแอมโมเนียมอะซิเตทบัฟเฟอร์:อะซิโตรไนไตรล์ วิธีการนี้ให้ความสามารถในการวัดซ้ำที่ต่ำ และให้ค่าการคืนกลับที่ไม่สามารถยอมรับได้ ซึ่งเป็นผลมาจากการเกิดปฏิกิริยาข้างเคียงของไฮดรอกซีลามีนและอะซิโตไนไตรล์ในเฟสเคลื่อนที่ วิธีที่สองคือเทคนิค HPLC กับคอลัมน์รีเวิร์สเฟสซึ่งทำการวิเคราะห์โดยใช้การเกิดอนุพันธ์อะซิโตนออกซีมระหว่างไฮดรอกซีลามีนและอะซิโตน การแยกเกิดขึ้นบนคอลัมน์ LiChroCART® (250 mm × 4 mm, RP-18 (5 µm)) โดยใช้เฟสเคลื่อนที่เป็น 10:90 (v/v) เมทานอล:น้ำ และเครื่องตรวจวัดชนิดยูวีวิสิเบิ้ลถูกตั้งที่ 230 nm วิธีนี้ให้ความเป็นเส้นตรงในช่วง 0.5-50.0 mg/L โดยมีความแม่นยำสูง (< 1.4%RSD) ความเข้มข้นต่ำสุดที่สามารถตรวจวัดได้ (DL) เท่ากับ 0.16 mg/L วิธีที่สามคือเทคนิคการวัดเชิงแสง เป็นการตรวจวัดไฮดรอกซีลามีนอย่างอ้อมโดยการรีดิวซ์เหล็ก(III) เป็นเหล็ก(II) และการเกิดสารประกอบเชิงซ้อนสีแดงของเหล็ก(II)-ออร์โท-ฟีแนนโทรลีน การดูดกลืนแสงของสารประกอบเชิงซ้อนนี้ถูกตรวจวัดโดยใช้เครื่องสเปกโทรโฟโตมิเตอร์ที่ 510 nm วิธีนี้ให้ความเป็นเส้นตรงในช่วง 0.05-2 mg/L และค่า DL เท่ากับ 0.003 mg/L ในขั้นสุดท้าย วิธีการวิเคราะห์ที่ถูกพัฒนาแล้วเหล่านี้จะถูกนำไปใช้ในการตรวจหาปริมาณไฮดรอกซีลามีนในตัวอย่างยาง

สำหรับตัวอย่างมิชลิน ได้การคืนกลับอยู่ที่ต่ำกว่า 2.9% เมื่อตัวอย่างถูกสกัดโดยวิธีการสกัดของแข็ง-ของเหลว และต่ำกว่า 13.5% เมื่อสกัดโดยวิธีสกัดร้อน สำหรับตัวอย่าง RTEC ตัวอย่างนี้แสดงค่าการคืนกลับที่สูงกว่าตัวอย่างมิชลิน โดยการคืนกลับอยู่ในช่วง 41.2-82.8% โดยวิธีการสกัดของแข็ง-ของเหลว และ 31.3-56.0% โดยวิธีสกัดร้อน

90 หน้า

CONTENTS

	Page
ACKNOWLEDGEMENTS	**iii**
ABSTRACT (ENGLISH)	**iv**
ABSTRACT (THAI)	**v**
LIST OF TABLES	**x**
LIST OF FIGURES	**xii**
LIST OF ABBREVIATIONS	**xvi**
CHAPTER I INTRODUCTION	**1**
CHAPTER II OBJECTIVES	**3**
CHAPTER III LITERATURE REVIEWS	**4**
3.1 Hydroxylamine	4
3.2 Natural rubber and applying hydroxylamine in rubber	5
3.3 Analytical techniques	7
3.3.1 Extraction methods	7
3.3.1.1 Solid-liquid extraction (SLE)	7
3.3.1.2 Soxhlet extraction	8
3.3.2 Derivatization and determination methods	9
3.3.2.1 HPLC technique	9
3.3.2.2 Spectrophotometry technique	11
CHAPTER IV MATERIALS AND METHODS	**15**
4.1 Instruments and equipment	15
4.1.1 HPLC apparatus	15
4.1.2 HPLC column	15
4.1.3 Spectrophotometer	16
4.1.4 Other instruments	16
4.2 Reagents and chemicals	17
4.3 Rubber samples	18

CONTENTS (cont.)

Page

4.4 Preparation of reagent solutions 18

4.4.1 Solutions for HPLC cation-exchange 18

4.4.1.1 Stock NH_2OH standard solution, 1000 mg/L 18

4.4.1.2 NH_2OH intermediate standard solution, 10 mg/L 18

4.4.1.3 Working NH_2OH standard solutions 18

4.4.1.4 Pyridine solution, 1000 mg/L 19

4.4.1.5 Ammonium acetate buffer pH 5.0 19

4.4.1.6 Mobile phase 19

4.4.2 Solutions for HPLC reversed-phase 19

4.4.2.1 Stock NH_2OH standard solution, 1000 mg/L 19

4.4.2.2 NH_2OH intermediate standard solution, 10 mg/L 19

4.4.2.3 Acetone oxime preparation 20

4.4.2.4 Mobile phase 20

4.4.3 Solutions for colorimetric method 21

4.4.3.1 Stock NH_2OH standard solution, 1000 and 10 mg/L 21

4.4.3.2 Ferric iron, 2×10^{-3} M 21

4.4.3.3 Ferrous iron, 1×10^{-3} M 21

4.4.3.4 Acetate buffer, pH 5 (500 mM acetic acid) 21

4.4.3.5 o-phenanthroline 1×10^{-3} M 21

4.4.3.6 Fe(II)-*o*-phenanthroline complex preparation 21

4.4.4 Sulfuric acid, 1.0 M 22

4.5 Procedures 22

4.5.1 Method optimization 22

4.5.1.1 HPLC with cation-exchange column 22

4.5.1.2 HPLC with reversed-phase column 23

4.5.1.3 Colorimetric method 23

CONTENTS (cont.)

Page

4.5.2 Rubber extraction method 24
4.5.2.1 Solid-liquid extraction 24
4.5.2.1.1 HPLC with cation-exchange 24
4.5.2.1.2 HPLC with reversed-phase 24
4.5.2.1.3 Colorimetric method 24
4.5.2.2 Reflux 25
4.5.3 Method validation 25
4.5.3.1 Specificity (selectivity) 25
4.5.3.2 Linearity 25
4.5.3.3 Detection limit (DL) and quantitation limit (QL) 26
4.5.3.4 Accuracy and precision 26

CHAPTER V RESULTS AND DISCUSSION 28

5.1 HPLC with cation-exchange column 28
5.1.1 Chromatographic optimization 28
5.1.1.1 Volume of organic fraction 29
5.1.1.2 Type of acetate salts in the mobile phase 31
5.1.1.3 Concentration of buffer 31
5.1.1.4 pH of the mobile phase 31
5.1.2 Analytical performances 35
5.2 HPLC with reversed-phase column 41
5.2.1 Chromatographic optimization 41
5.2.1.1 Column selection 42
5.2.1.2 Mobile phase composition 43
5.2.2 Analytical performances 45
5.3 Colorimetric method 47
5.3.1 Absorption spectrum of Fe(II)-*o*-phenanthroline 47
5.3.2 Effect of pH 48

CONTENTS (cont.)

	Page
5.3.3 Kinetic study of the complex	49
5.3.4 Reducing power of NH_2OH	51
5.3.5 Job's plot	51
5.3.6 Analytical performances	52
5.4 Hydroxylamine in rubber samples	55
5.4.1 Solid-liquid extraction (SLE)	55
5.4.2 Reflux	56
5.4.3 Summary	57
CHAPTER VI CONCLUSION	**59**
REFERENCES	**61**
APPENDICES	**67**
BIOGRAPHY	**90**

LIST OF TABLES

Table		Page
3.1	Percentage weight of fresh latex component.	6
4.1	Other instruments used in the laboratory.	16
4.2	List of chemicals and suppliers.	17
4.3	The procedure of adding solution for acetone oxime formation.	20
5.1	Analytical data of the HPLC cation-exchange method for standard NH_2OH under the optimum conditions.	36
5.2	Method precision and recovery for chromatographic analysis of standard NH_2OH added into aqueous system (n = 10).	36
5.3	Analytical data of the HPLC reversed-phase method for standard NH_2OH under the optimum conditions.	46
5.4	Method precision and recovery for chromatographic analysis of standard NH_2OH added into aqueous system (n = 10).	46
5.5	Analytical data of the colorimetric method for standard NH_2OH under the optimum conditions.	53
5.6	Method precision and recovery for the colorimetric analysis of standard NH_2OH added into aqueous system (n = 10).	54
5.7	Measured concentrations of $(NH_2OH)_2.SO_4$ in Michelin and RTEC samples extracted by SLE.	56
5.8	Measured concentrations of $(NH_2OH)_2.SO_4$ in Michelin and RTEC samples extracted by reflux.	57
5.9	The summary of NH_2OH analysis analyzed from Michelin samples.	58
5.10	The summary of NH_2OH analysis analyzed from RTEC samples.	58
A.1	Data of NH_2OH analysis in rubber samples using HPLC with cation-exchange method.	80
A.2	Data of NH_2OH analysis in rubber samples using HPLC with reversed-phase column.	81
A.3	Data of NH_2OH analysis in rubber samples using colorimetric method (SLE).	82
A.4	Homogeneity check of rubber samples.	84

LIST OF TABLES (cont.)

Table		Page
A.5	The results and the calculation for DL.	86
A.6	The experiment for confirming QL.	86
A.7	The statistic calculation for confirming QL.	87
A.8	The acceptable recovery requirements from AOAC.	87
A.9	RSD_R calculated from different concentrations of analyte.	88
A.10	Example for the calculation of HORRAT.	89

LIST OF FIGURES

Figure		Page
3.1	Chemical structure of isoprene and polyisoprene.	5
3.2	The reaction schemes of hydroxylamine and vanillin.	10
3.3	The reaction schemes of 'hydroxylamine and Fe^{3+}' and the reaction schemes of $[Fe(Ferrozine)_3]^{4-}$ complex formation.	11
3.4	The reaction schemes of 'hydroxylamine and sodium arsenate' and the reaction schemes of 'the diazocoupling reaction between *p*-nitroaniline and *N*-(1-naphthyl)ethylenediamine'.	12
3.5	The reaction schemes of 'hydroxylamine and iodate' and the reaction schemes of 'the diazocoupling reaction between *p*-nitroaniline and N,N-dimethylaniline'.	12
3.6	The reaction schemes of 'hydroxylamine and iodate' and the reaction schemes of 'nitrite ion and neutral red'.	13
3.7	The reaction schemes of 'hydroxylamine and bromine' and the reaction schemes of 'bromine and methyl red'.	14
5.1	HPLC chromatograms of 10 mg/L of pyridine and 20 mg/L of NH_2OH using various ratio of ammonium acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile; (a) 60:40 (%v/v), (b) 70:30 (%v/v), (c) 80:20 (%v/v), and (d) 90:10 (%v/v).	30
5.2	HPLC Chromatogram of 10 mg/L of pyridine and 20 mg/L of NH_2OH using 60:40 (%v/v) acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile that was prepared from different types of acetate salt; (a) ammonium acetate salt and (b) sodium acetate salt.	32

LIST OF FIGURES (cont.)

Figure		Page
5.3	HPLC chromatograms of 10 mg/L of pyridine and 20 mg/L of NH_2OH using 60:40 (%v/v) ammonium acetate buffer pH 4.5:acetonitrile as the mobile phase, varying the concentration of CH_3COOH (mM):CH_3COONH_4 (mM); (a) 5:2.7, (b) 10:5.5, (c) 20:11, and (d) 30:16.5.	33
5.4	HPLC chromatograms of 10 mg/L of pyridine and 20 mg/L of NH_2OH using 60:40 (%v/v) ammonium acetate buffer (10 mM acetic acid) with various pH values:acetonitrile; (a) pH 4.0, (b) pH 4.5, (c) pH 5.0, and (d) pH 5.5.	34
5.5	Calibration curve of standard NH_2OH for the HPLC cation-exchange method.	36
5.6	The increase of peak area with time from measuring the same solution of 10 mg/L NH_2OH.	37
5.7	3-Days monitoring of the quantity of NH_2OH dissolved in mediums of water and mobile phase.	38
5.8	The reaction schemes of NH_2OH and acetonitrile.	39
5.9	HPLC chromatograms of 10 mg/L of pyridine and 30 mg/L of NH_2OH using different organic solvent; (a) 60:40 (%v/v) acetate buffer pH 4.5:acetonitrile, and (b) 60:40 (%v/v) acetate buffer pH 4.5:methanol.	40
5.10	The reaction schemes of NH_2OH and acetone.	41
5.11	HPLC chromatograms of acetone and acetone oxime (50 mg/L of NH_2OH) using different types of column; (a) column Nova-Pak® C18 (150 mm × 3.9 mm), and (b) LichroCART® C18 (250 mm × 4 mm).	43

LIST OF FIGURES (cont.)

Figure		Page
5.12	HPLC chromatograms of acetone and acetone oxime (prepared from 50 mg/L of NH_2OH) using different mobile phase proportion; (a) 25:75 (%v/v), (b) 20:80 (%v/v), (c) 15:85 (%v/v), and (d) 10:90 (%v/v) of methanol:DI water.	44
5.13	Calibration curve of standard NH_2OH for the HPLC reversed-phase method.	45
5.14	The reaction schemes of the formation of Fe(II)-*o*-phenanthroline complex.	47
5.15	Absorption spectrum of Fe(II)-*o*-phenanthroline.	48
5.16	Effect of pH on the formation of Fe(II)-*o*-phenanthroline complex.	49
5.17	The kinetic study of Fe(II)-*o*-phenanthroline complex formation at different pH; (a) 4.0, (b) 4.5, (c) 5.0, and (d) 5.5.	50
5.18	Reducing power of NH_2OH for donating electron in reduction reaction of Fe^{3+}.	51
5.19	Job's plot showing the 1:3 stoichiometric mole ratio of the complexation between Fe^{2+} and o-phenanthroline.	52
5.20	Calibration curve of standard NH_2OH for the colorimetric method.	53
A.1	Production procedures of CV rubber by using natural rubber latex.	68
A.2	Production procedures of CV rubber by using natural rubber cup lump.	68
B.1	HPLC chromatograms of 10 mg/L of pyridine and 50 mg/L of NH_2OH using different mobile phase: (a) 10 mM nitric acid; (b) 10 mM acetic acid; (c) 10 mM sulfuric acid; (d) 10 mM phosphoric acid; (e) 60:40 (%v/v) acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile.	69
C.1	Absorption spectrum (in range 200-600 nm) of 50 mg/L of NH_2OH in 60:40 (%v/v) acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile after set zero with solvent blank.	70
C.2	Absorption spectrum (in range 200-400 nm) of pyridine in DI water.	70

LIST OF FIGURES (cont.)

Figure		Page
C.3	Absorption spectrum scanning in the range of 200-300 nm of acetone oxime prepared from NH_2OH 100 mg/L.	71
D.1	Effect of reaction temperature to the formation of acetone oxime.	72
D.2	Effect of reaction time to the formation of acetone oxime.	72
E.1	Optimization of colorimetric method by varing of different reagents (a) Fe^{3+}, (b) o-phenanthroline, (c) hydroxylamine, and (d) acetate buffer.	73
F.1	Natural rubber sample during solid-liquid extraction	74
G.1	Picture of reflux set-up for extraction of natural rubber	75
H.1	Chromatograms of Michelin samples analysis obtained from HPLC with cation-exchange column.	76
H.2	Chromatograms of Michelin samples analysis obtained from HPLC with reversed-phase column.	77
I.1	Chromatograms of RTEC samples analysis obtained from HPLC with cation-exchange column.	78
I.2	Chromatograms of RTEC samples analysis obtained from HPLC with reversed-phase column.	79
K.1	The degradation process of NH_2OH in 3 g/kg RTEC rubber.	83
L.1	Homogeneous of NH_2OH in RTEC rubber sample	84

LIST OF ABBREVIATIONS

µL	Microliter
µm	Micrometer
µM	Micromolarity
ºC	Temperature in degree of Celsius
AOAC	Association of Official Analytical Chemists
AR	Analytical reagent
cm	Centimeter
CV	Constant viscosity
DI	Deionized
DL	Detection limit
DRC	Dry rubber content
e.g.	Exempli gratia (Latin), for example
et al.	Et. Alli (Latin), and others
etc.	Et cetara (Latin), and so on
g	Gram
GC	Gas Chromatography
GPS	Global Product Strategy
HNS	Hydroxylamine sulfate
HPLC	High Performance Liquid Chromatography
hr	Hour
i.e.	Id est (Latin), that is
IC	Ion chromatography
ICCA	International Council of Chemical Associations
IR	Infrared
kg	Kilogram
L	Liter

LIST OF ABBREVIATIONS (cont.)

M	Molarity
mg	Milligram
mg/L	Milligram per liter
min	Minute
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
mM	Millimolarity
mol	mole
n	Number of analysis
nm	Nanometer
NR	Natural rubber
NRC	Non rubber content
pH	Potential of Hydrogen ion
QL	Quantitation limit
RP	Reversed phase
R_s	Resolution
RSD	Relative standard deviation
RTEC	Rubber Technology Research Centre
SD	Standard deviation
SLE	Solid-liquid extraction
STR	Standard Thai Rubber
t	Time
UV	Ultraviolet
v/v	Volumn by volume
W	Width

CHAPTER I
INTRODUCTION

Hydroxylamine or NH_2OH is an inorganic compound in group of a strong reducing agent that is useful in organic synthesis (PubChem, 2016). In the industrial scale, NH_2OH and its derivatives are applied in several chemical industries, such as, a therapeutic agent in drug synthesis (Fernando, Egwu, & Hussain, 2002), a stabilizer of fatty materials in the production of soap (Scherr, 1948), a preservative for liquid color developer (Abe, 1998), etc. Moreover, in the rubber industry, NH_2OH has been used as a "viscosity stabilizer" for controlling viscosity of natural rubber (NR) during storage.

When stored NR for a long month, it is found to be progressively harder or increase in viscosity. This phenomenon is called "storage hardening" that is the result from the formation of the linkage between rubber molecules and non rubber molecules. The linkage will cause the difficulty in mobility of the molecular chains (Ngolemasango et al., 2003). Storage hardening will be a problem for the industrial process to handle harder rubber because it requires more production energy. Therefore, in the rubber processing industries, the "constant viscosity rubber" or "CV rubber" must be prepared by treating with various chemicals in group of monofunctionaldehydic condensing reagents, such as hydroxylamine hydrochloride, hydroxylamine sulphate, and semicarbazide hydrochloride. These compounds will prevent the linkage between molecules and then stabilize the viscosity of NR (ทัศนกุล, 2557). However, the use of NH_2OH in NR have to be controlled in the optimum conditions because using different amounts of NH_2OH will produce different viscosities of CV rubber (Yongzhou et al., 2012). Therefore, the quantity of NH_2OH in CV rubber must be measured before used to investigate the quality of CV rubber.

In previous researches, various analytical methods had been reported for the determination of NH_2OH, such as, gas chromatography (Guzowski Jr, Golanoski, & Montgomery, 2003; Peng et al., 1999; Seike et al., 2004), high performance liquid

chromatography (Korte, 1992), spectrophotometry (Afkhami, Madrakian, & Maleki, 2005, 2006; Deepa, Balasubramanian, & Nagaraja, 2004; Dias, Olojola, & Jaselskis, 1979; George, Balasubramanian, & Nagaraja, 2007), ion chromatography with electrochemical detector (Fernando et al., 2002; Prokai & Ravichandran, 1994) and capillary ion electrophoresis (Bowman, Tang, & Silverman, 2000). These methods were applied to different samples, such as, pharmaceutical samples, water samples, biological samples, etc. However, there is no research presenting the analytical method for NH_2OH analysis in rubber sample. Therefore, the aim of this research is to develop the analytical method and sample preparation method for the analysis of NH_2OH in NR.

This project was conducted by the cooperation between Michelin company and Rubber Technology Research Center (RTEC), Mahidol University. The developed analytical and sample preparation methods were performed on the basis of simple, selective and effective for NH_2OH analysis in rubber sample. Three analytical methods were developed and optimized to detect NH_2OH; (1) high performance liquid chromatography (HPLC) with cation-exchange column, (2) HPLC with reversed-phase column, and (3) colorimetry using Fe(II)-*o*-phenanthroline complex formation. The natural rubber samples in this work were obtained from Michelin and RTEC. Two extraction methods were used for extracting NH_2OH, i.e., solid-liquid extraction and reflux.

CHAPTER II
OBJECTIVES

The aims of this thesis are shown below;

1. To develop the analytical method for the determination of NH_2OH compound by high performance liquid chromatography (HPLC) and spectrophotometry

2. To study the extraction method, i.e., solid-liquid extraction and reflux, for extracting NH_2OH from rubber samples

3. To apply the developed method for the determination of NH_2OH in natural rubber samples obtained from Michelin company and Rubber Technology Research Center (RTEC)

CHAPTER III
LITERATURE REVIEWS

This chapter will summarize the literature reviews about the information of (1) hydroxylamine (NH_2OH) and the applying NH_2OH in natural rubber, (2) analytical methods including extraction, derivatization, and determination techniques.

3.1 Hydroxylamine

Hydroxylamine (NH_2OH) is a colorless inorganic compound that has been classified as a strong reducing agent. At room temperature, pure hydroxylamine is an unstable hygroscopic compound that can rapid decompose and tend to be explosive (PubChem, 2016). Therefore, hydroxylamine is mostly preserved in salt forms, such as, hydroxylamine sulfate, hydroxylamine hydrochloride, etc.

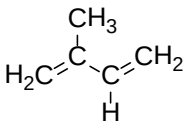
Hydroxylamine and its salts are applied in many branches of chemical industries. For example, hydroxylamine is used as a raw material in the synthesis of pharmaceutical intermediates and final drug substances (Fernando, Egwu, & Hussain, 2002), used in soap production as an effective stabilizer on the fatty materials (Scherr, 1948), used as a preservative in liquid color developer (Abe, 1998), can use to cleave asparaginyl-glycine peptide bonds in the fusion protein and the antimicrobial peptide (Park, Pyo, Hong, & Kim, 2001), etc. However, hydroxylamine can be toxic to human health and environment.

In health concern, hydroxylamine is moderately toxic to human body. With a short term exposure, hydroxylamine can be harmful to digestive and respiratory tract and also irritate to eyes and skin. With a long term exposure, hydroxylamine can cause a skin sensitization and can affect blood system. Furthermore, the use in the industry will release waste of hydroxylamine to an environment. From the environmental information, hydroxylamine was considered to be toxic to aquatic organisms. (The safety information refers to Global Product

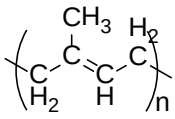
Strategy (GPS) launched by The International Council of Chemical Associations (ICCA) (BASF, 2012).)

3.2 Natural rubber and applying hydroxylamine in rubber

Natural rubber obtained from fresh latex is a colloidal suspension of rubber particle dispersed in a liquid called “serum”. The rubber particle is a polymeric hydrocarbon with a chemical name as 1,4-polyisoprene that consists of nearly 100% *cis*- structure (*cis*-1,4-polyisoprene). Polyisoprene is made from many binding isoprene monomers that comprise 5 atoms of carbon (C) and 8 atoms of hydrogen (H). The chemical structures of isoprene and polyisoprene are shown in Figure 3.1.



Isoprene



cis-1,4-polyisoprene

Figure 3.1 Chemical structure of isoprene and polyisoprene.

The composition of fresh latex devided into 2 parts that is

(1) Dry rubber content (DRC) is the rubber particle of binding isoprene about 2000–5000 units per molecule.

(2) Non rubber content (NRC) is the other non rubber components, such as, glucoses, proteins, fats, carotenoids, minerals, enzymes, and nitrogen compounds

The amount of each component in fresh latex is shown in Table 3.1. However, the components of fresh latex are inconstant. It is depend on rubber species, rubber age, tapping period or tapping process, etc. (พัฒนกุล, 2554)

Table 3.1 Percentage weight of fresh latex component.

Component	Percent by weight
All solid (rubber and non rubber)	36 (33% DRC and 3% NRC)
Proteins	1-1.5
Resins	1-2.5
Ashes	nearly 1
Glucose	1
Water and other compounds	

During storage, natural rubber can undergo “storage hardening” or increases in the bulk viscosity because of the formation of the linkage between the rubber molecules and non rubber molecules. The mentioned mechanism is about the interactions between abnormal groups on rubber molecules (such as, aldehydes, epoxies, lactones, and esters on the polyisoprene chains) and non rubber molecules (such as, amino acids or proteins and certain lipids). Storage hardening will be exacerbated if natural rubber is stored at low temperature for several months. Therefore, to avoid this effect, natural rubber is obliged to store in heated warehouses. (Ngolemasango et al., 2003). However, in the industrial scale, the viscosity of natural rubber must be controlled because using harder rubber will require more production energy. Therefore, there is the use of the modified natural rubber called “constant viscosity rubber” or “CV rubber”.

The CV rubber is natural rubber prepared to have constant viscosity by adding the chemical in group of monofunctionaldehydic condensing reagents, such as, hydroxylamine hydrochloride, hydroxylamine sulphate, and semicarbazide hydrochloride. These compounds have been called “viscosity stabilizer”. The role of these chemicals is to react with the abnormal groups on the rubber molecules in order to prevent the formation of bond linkage between molecules. (Yongzhou et al., 2012; Zhang et al., 2012). The usability of CV rubber has been occurred in various rubber processing industries, such as, rubber hose industry, glue industry, tyre industry, etc. The quality grade of CV rubber can be divided into three levels depend on STR grade (Standard Thai Rubber); STR 5 CV, STR 10 CV and STR 20 CV (ทัศนกุล, 2557).

The production processes of CV rubber were devided into two methods according to the material of natural rubber used, such as, latex and cup lump (the details of the process are described in Appendix A). Latex is a stable emulsion of polyisoprene microparticles in an aqueous medium. Cup lump is the latex that is coagulated into a lump in the bottom of a cup. In each method, the amount of hydroxylamine used was 0.15% by weight for latex and 0.4% by weight for cup lump (Thaimac, 2015; มหาวิทยาลัยสงขลานครินทร์, 2553).

3.3 Analytical techniques

From the literature review of the previous researches, there was no report clearly indicating the procedure or the method to analyze hydroxylamine in rubber. Therefore, we devided the literature review into two sections including (1) extraction methods for rubber samples and (2) the technique for derivatization and determination of hydroxylamine.

3.3.1 Extraction methods

In the analysis of solid sample, the interested analyte must be removed from the solid sample into a liquid extraction solution that is suitable for the analytical techniques used and also can reduce the matrix interference from the sample. The extraction methods commonly used for rubber sample are solid-liquid extraction (or shake-filter method) and soxhlet extraction.

3.3.1.1 Solid-liquid extraction (SLE)

Ikarashi and Kaniwa (Ikarashi & Kaniwa, 2000) used the SLE method for extracting *p*-phenylenediamine from rubber boot. First, 1.0 g of sample was cut into small size, about 1 mm × 10 mm, and placed in a glass centrifuge tube. Then 10 mL of (1:1) acetone:chloroform was added. The solution was shaken for 30 min at room temperature. The liquid phase was filtrated and collected in round-bottomed flask. Three times extractions were performed to reextract the residue. The

extracts were evaporated using a vacuum rotary evaporator at 50 ºC. Finally, the extract was dissolved in 1 mL of dichloromethane and then added to a glass column.

In 2004 and 2006, Bergendorff et al. used the same SLE method to extract alkyl thioureas from an orthopaedic brace and patch (Bergendorff, Persson, & Hansson, 2004) and extract rubber allergen from glove (Bergendorff, Persson, & Hansson, 2006). First, 0.5 g of sample was cut into small pieces and placed in 10 mL test tube with a Teflon-lined screw cap. Then the test tube was added with 5 mL of acetone and shaken for 10 min at room temperature. The extract was separated and evaporated under vacuum in a round-bottomed flask. Finally, the extract was dissolved in 5 mL acetonitrile and injected to HPLC column.

Avagyan, et al. (Avagyan, Sadiktsis, Thorsén, Östman, & Westerholm, 2013) extracted benzothiazole and its derivatives from tire by using SLE method with various extraction solvent. First, 0.2 g of tire particle was placed in 15 mL test tube with Teflon-lined screw caps and then 10 mL of extraction solvent was added. The tube was immersed in an ultrasonic bath for 1 hr. The extract was filtrated using 0.2 µm nylon filters and transferred to new test tube. The residue was reextracted with fresh solvent. The combined extract was evaporated under a nitrogen stream to 0.2 mL. The extract was then mixed with 0.3 mL of methanol and then filtrated again before injecting to HPLC.

3.3.1.2 Soxhlet extraction

Blanco, et al. (Blanco, Coello, Iturriaga, Maspoch, & Bertran, 1997) used Soxhlet extraction method to extract phenyl-*β*-naphthylamine from rubber. The vulcanized rubber sheets were cut into small pieces, 0.5 cm × 0.5 cm and then treated with 75 mL of carbon tetrachloride in a Soxhlet apparatus for 6-8 hr. The extract was dried to 5 mL using IR beam lamp and then applied to infared spectrophotometry.

Guerra, et al. (Guerra, Marín, Sánchez, & Jiménez, 2002) applied Soxhlet extraction method for extracting citrates and benzoates from poly(vinyl chloride). In their work, 2 g of samples were refluxed with 70 mL of organic solvent for 5 hr in Soxhlet apparatus. Then, the extracts were determined by GC.

In 2002 and 2005, Moldovan *et al*. presented the use of Soxhlet extraction method in two researches that are spectophotometric (Moldovan & Alexandrescu, 2002) and spectrofluorimetric (Moldovan, Stoica, Hillebrand, Alexandrescu, & MacOvescu, 2005) determination of phenyl-*β*-naphthylamine in rubber mixtures. The extraction was performed by heating 2.5 g of small piece rubber with acetone under reflux for 24 hr. The system was done under nitrogen atmosphere to avoid the decomposition of the analyte. The extract was evaporated and dried with vacuum. 0.5 mL of the extract was diluted to 25 mL with chloroform and measured the spectra of the solution.

3.3.2 Derivatization and determination methods

In the last ten years, the researches about hydroxylamine analysis were mostly presented in the field of 'electrochemical sensor using modified electrode' (Benvidi, Jahanbani, Akbari, & Zare, 2015; Benvidi et al., 2013; Foroughi, Beitollahi, Tajik, Hamzavi, & Parvan, 2014; Shabani-Nooshabadi & Tahernejad-Javazmi, 2015; Shishehbore, Zare, Nematollahi, & Saber-Tehrani, 2011). Other works were about chromatographic and spectrophotometric technique, i.e., high performance liquid chromatography (Fernando et al., 2002; Korte, 1992; Prokai & Ravichandran, 1994), gas chromatography (Guzowski Jr, Golanoski, & Montgomery, 2003; Peng et al., 1999; Seike et al., 2004) and spectrophotometry (Afkhami, Madrakian, & Maleki, 2005, 2006; Deepa, Balasubramanian, & Nagaraja, 2004; Dias, Olojola, & Jaselskis, 1979; George, Balasubramanian, & Nagaraja, 2007). Some works showed the use of flow injection analysis (Bourke, Stedman, & Wade, 1983) and capillary electrophoresis (Bowman, Tang, & Silverman, 2000). In each technique, derivatization and non-derivatization of hydroxylamine was performed.

This section summarizes the literature reviews of derivatization reactions for converting NH_2OH to form a suitable analyte compound for the instrument used, i.e., HPLC and spectrophotometer.

3.3.2.1 HPLC technique

Korte (Korte, 1992) proposed a high performance liquid chromatography using UV and fluorescence detection for determining hydroxylamine

in form of vanillin oxime derivative in aqueous systems. The derivatization reaction of vanillin oxime is shown in Figure 3.2. The compound was separated on a reversed-phase 10-µm µBondapak C18 column (30 cm × 3.9 mm) by using the mixture of acetonitrile and potassium phosphate buffer as a mobile phase. The HPLC method measured hydroxylamine in concentration range 5×10^{-2} to 5×10^{-5} M with RSD 1 to 2%. The accuracy of the method was observed at 92 to 95%.

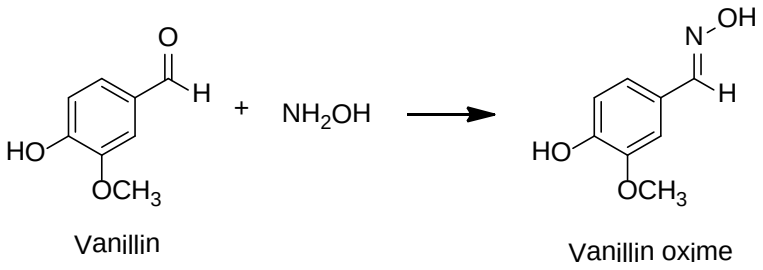


Figure 3.2 The reaction schemes of hydroxylamine and vanillin.

Prokai and Ravichandran (Prokai & Ravichandran, 1994) presented the direct analysis of hydroxylamine and N-alkylhydroxylamines by using ion chromatography with conductivity and amperometric detectors. The column used was Alltech/Wescan Cation/R (150 mm × 4.6 mm) and elution solvent was 10 mM nitric acid. However, the effluent must be adjusted to achieve the alkaline pH before going to electrochemical detector using post-column addition of a strong base. Linear range of hydroxylamine was 0.02 to 0.5 mM and Detection limit (DL) was 0.01 mM for conductivity detector and linear in the range of 0.001 to 0.05 mM with DL at 0.0002 mM for amperometric detector.

Fernando, et al. (Fernando et al., 2002) reported a cation-exchange chromatographic method with pulsed amperometric detection using gold electrode for the direct analysis of trace hydroxylamine in pharmaceutical waste stream. The cation separation was achieved by an IonPac CS14 (250 mm × 4 mm) analytical column with 11 mM sulfuric acid eluent. The post-column base (300 mM NaOH) was used. The obtained hydroxyalmine signal showed the linear response in range 0.05 to 2.0 mg/L with DL at 0.0015 mg/L. The recoveries of hydroxylamine in sample matrix were 69.4% (0.05 mg/L) and 93.3% (0.5 mg/L).

3.3.2.2 Spectrophotometry technique

Dias, et al. (Dias et al., 1979) presented spectrophotometric determination of hydrazine and hydroxylamine by using the reaction with Fe^{3+} in the presence of Ferrozine (Figure 3.3). The $[Fe(Ferrozine)_3]^{4-}$ complex was formed at the control pH 3.2. The complex was detected by spectrophotometer at 562 nm and the calibration curve was plotted in the concentration range of 4×10^{-6} to 40×10^{-6} M of hydroxylamine. Apparent molar absorptivity was $3.67 \pm 0.02 \times 10^4$ $l.mole^{-1}.cm^{-1}$.

$$2Fe^{3+} + 2NH_2OH{\cdot}HCl + 2OH^- \longrightarrow 2Fe^{2+} + N_2 + 4H_2O + H^+ + Cl^-$$

$$Fe^{2+} + 3Ferrozine \longrightarrow Fe(Ferrozine)_3^{4-}$$

Figure 3.3 The reaction schemes of 'hydroxylamine and Fe^{3+}' and the reaction schemes of $[Fe(Ferrozine)_3]^{4-}$ complex formation.

Deepa, et al. (Deepa et al., 2004) developed a spectrophotometric method for determing hydroxylamine and its derivatives in pharmaceutical formulations. The method started from the oxidation of hydroxylamine to nitrite using sodium arsenate. Nitrite product will be detected by using the diazo coupling reaction between *p*-nitroaniline and *N*-(1-naphthyl)ethylenediamine (Figure 3.4). The azo dye compound was measured at 545 nm. The linearity was shown in the concentration range of 0 to 0.28 mg/L and the molar absorptivity was 6.7×10^4 $l.mole^{-1}.cm^{-1}$.

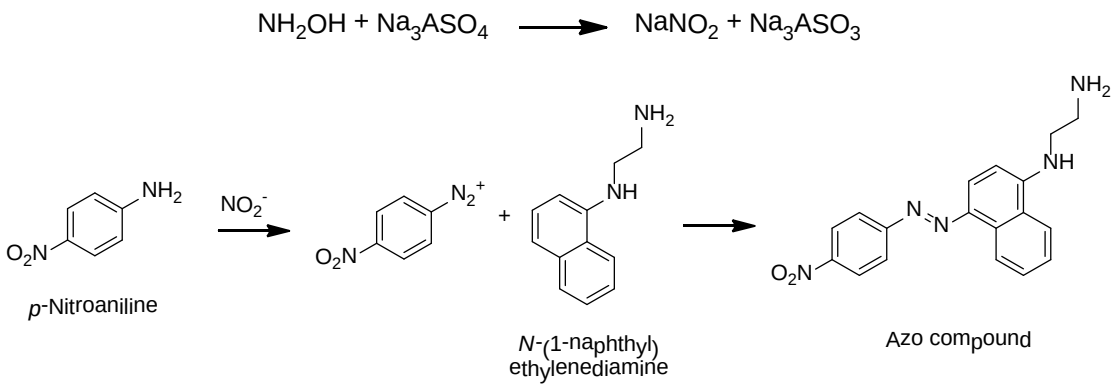


Figure 3.4 The reaction schemes of 'hydroxylamine and sodium arsenate' and the reaction schemes of 'the diazocoupling reaction between *p*-nitroaniline and *N*-(1-naphthyl)ethylenediamine'.

Afkhami, et al. (Afkhami et al., 2005) proposed the colorimetric method for hydroxylamine analysis based on the reaction of hydroxylamine with iodate to produce nitrite ion. The nitrite ion will proceed to occur the diazo coupling reaction with *p*-nitroaniline and N,N-dimethylaniline and produce azo compound (Figure 3.5). The spectrophotometric measurement at 494 nm was used. The method showed linear in the range of 0.21 to 6.06 µM of hydroxylamine with DL at 0.046 µM. The application of this method in water sample and biological sample showed the recovery close to 100%.

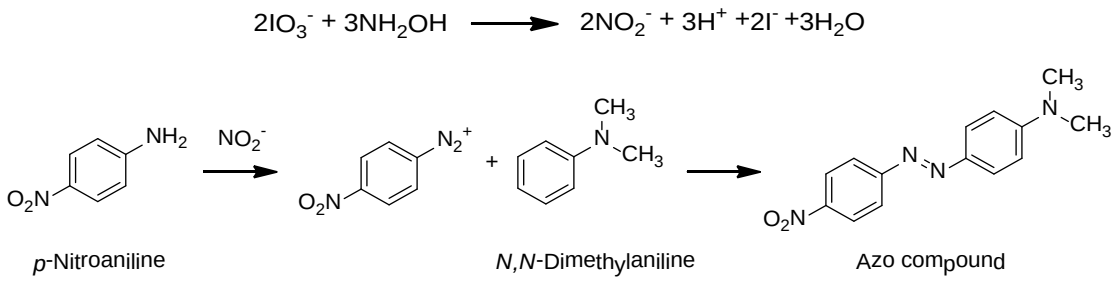


Figure 3.5 The reaction schemes of 'hydroxylamine and iodate' and the reaction schemes of 'the diazocoupling reaction between *p*-nitroaniline and N,N-dimethylaniline'.

In the next year, Afkhami, et al. (Afkhami et al., 2006) proposed another derivatization reaction of nitrite ion by using the reaction with neutral red. This method will detect the decrease in the absorbance of the solution. The reaction showed in Figure 3.6. The occurrence was monitored by spectrophotometer at 525 nm by a fixed time method. Linear range of hydroxylamine was 0.040 to 1.200 mg/L with DL at 0.010 mg/L. The precision (%RSD) from ten determinations of 0.500 mg/L hydroxylamine was at 1.81%. This method was applied for determining hydroxylamine in water samples with recoveries close to 100%.

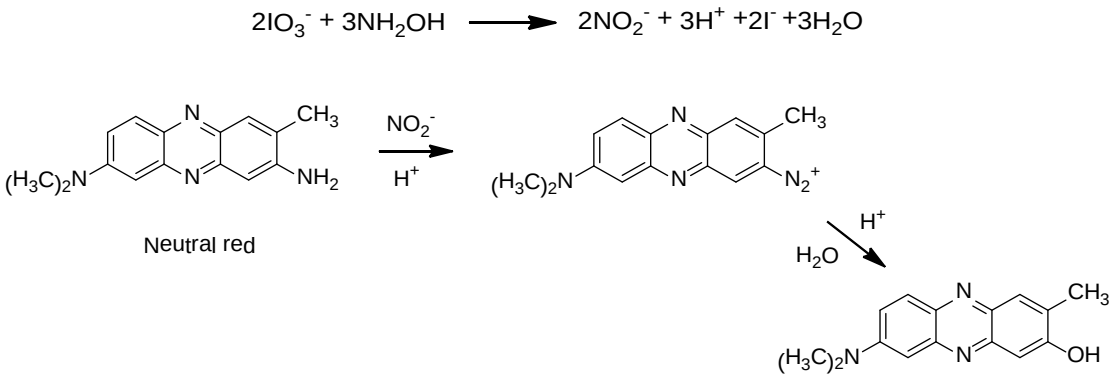


Figure 3.6 The reaction schemes of 'hydroxylamine and iodate' and the reaction schemes of 'nitrite ion and neutral red'.

George, et al. (George et al., 2007) presented a colorimetric method using methyl red for the determination of hydroxylamine and its derivatives in drug formulations. First, they used the reaction between hydroxylamine and a known excess of bromine to produce bromide ion and then the unreacted bromine will bleach the color of dye methyl red (Figure 3.7). The reaction was monitored by spectrophotometer at 520 nm. This method obeys Beer's law in the range of 0 to 0.2 mg/L with molar absorptivity at 9.80×10^4 $l.mol^{-1}.cm^{-1}$. RSD from detecting 0.12 mg/L of hydroxylamine was 2.7%.

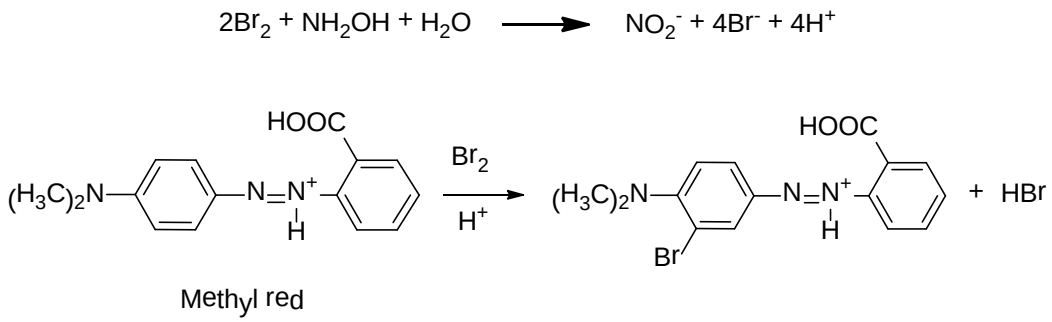


Figure 3.7 The reaction schemes of ‘hydroxylamine and bromine’ and the reaction schemes of ‘bromine and methyl red’.

CHAPTER IV
MATERIALS AND METHODS

The details of the experimentation are shown in this chapter, such as, instruments and equipment, reagents and chemicals, rubber samples, reagent preparation, and experimental procedures.

4.1 Instruments and equipment

4.1.1 HPLC apparatus

The model of HPLC instrument was Waters LC Module I plus (Minnesota, United States) consist of:

Pump : Multisolvent delivery system, Waters 600 HPLC pump
Injector : Waters 715 Autosampler
Detector : Waters 486 Tunable Absorbance Detector
Software : Millennium 32

4.1.2 HPLC column

Synchrom S300 column (250 mm × 4.6 mm); a polymeric, hydrophilic, silica-based strong cation-exchange support; supplied by Eichrom Technologies (Illinois, USA) was used in cation-exchange chromatographic separation of NH_2OH and pyridine in rubber.

LiChroCART® (250 mm × 4.6 mm, RP-18 (5 µm)); a spherical silica gel carrier with C-18; supplied by Merck Millipore (Darmstadt, Germany) was used in reversed-phased chromatography for the separation of acetone and acetone oxime in rubber.

4.1.3 Spectrophotometer

The absorbance measurement of solutions was carried out by using UV-Visible spectrophotometer (Shimadzu UV-2600) with a 1.0 cm quartz cuvette; Hellma quartz cuvette (absorption cells); 45 × 12.5 × 12.5 mm, light path 10 mm, volume 3500 µL (Müllheim, Germany).

4.1.4 Other instruments

Other instruments and equipment are summarized in the Table 4.1.

Table 4.1 Other instruments used in the laboratory.

Instrument	Model	Manufacturer
Analytical balance	Sartorius CP 225D	Goettingen, Germany
Autopipette	Eppendorf Research® plus	Hamburg, Germany
pH meter	Denver model 215	Pennsylvania, USA
Rotary vacuum pump	2XZ-2C	Zhejiang, China
Ultrasonic bath	NEY Model ULTRAsonik 280H	Yucaipa CA, USA
Agitator vortex	Nahita 681	Navarra, Spain
Reciprocating shaker	GFL 3006 series	Burgwedel, Germany
Magnetic stirring hotplate	Heidolph 3001 series	Schwabach, Germany
Low temperature circulator cool ace	Eyela CA-1111	Bunkyo-ku, Japan
Rolling mill	COLLIN W 100 T	Ebersberg, Germany

4.2 Reagents and chemicals

In this work, various chemicals and solvents from different supplier were used for standard and sample preparations. The list of these chemicals is shown in Table 4.2.

Table 4.2 List of chemicals and suppliers.

Reagents and chemicals	Suppliers
Acetic acid glacial (99.7%)	Lab-scan (Thailand)
Acetone (AR Grade)	Lab-scan (Thailand)
Acetonitrile (HPLC grade)	Burdick&Jackson (Korea)
Ammonium acetate (98%)	Merck (Germany)
Ammonium iron (II) sulfate (Extra pure)	Merck (Germany)
Ammonium iron (III) sulfate	UNIVAR (USA)
Chloroform	Lab-scan (Thailand)
Dichloromethane (AR Grade)	Lab-scan (Thailand)
Hexane	Burdick&Jackson (Korea)
Hydroxylamine sulfate (99%)	Sigma-Aldrich (USA)
Methanol (HPLC grade)	Burdick&Jackson (Korea)
Nitric acid (70%)	Merck (Germany)
o-phenanthroline	Scharlau (Spain)
Pyridine	LOBAL Chemie (India)
Sodium acetate (99%)	Merck (Germany)
Sulfuric acid	Merck (Germany)
Toluene	Lab-scan (Thailand)

4.3 Rubber samples

The rubber samples in this work were obtained from Michelin company and Rubber Technology Research Centre (RTEC). Natural rubbers were treated with hydroxylamine sulfate (HNS) at different concentration of 0.0, 0.5, 0.8, 2, and 3 g HNS/1 kg rubber. The Michelin rubber samples were prepared by spraying hydroxylamine sulfate dissolved in water on the rubber with a syringe and then mixed using rolling mill to disperse hydroxylamine in the dried natural rubber. The RTEC rubber samples were prepared by strewing hydroxylamine sulfate powder on the dried natural rubber and then mixed well with rolling mill.

4.4 Preparation of reagent solutions

The preparation procedures for reagent solutions in each method were described separately following the analytical method used.

4.4.1 Solutions for HPLC cation-exchange

4.4.1.1 Stock NH_2OH standard solution, 1000 mg/L

0.0621 g of hydroxylamine sulfate was weighed and then dissolved in mobile phase; 60:40 (%v/v) ammonium acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile. The solution was transferred into a 25.00 mL volumetric flask and made up to volume with mobile phase. This solution will be used as a stock standard solution to make other dilution.

4.4.1.2 NH_2OH intermediate standard solution, 10 mg/L

The NH_2OH intermediate standard at 10 mg/L was prepared by transferring 100.0 µL of 1000 mg/L stock NH_2OH standard solution into a volumetric flask 10.00 mL and then adjusted to volume with the mobile phase used.

4.4.1.3 Working NH_2OH standard solutions

Working NH_2OH standard solution at concentrations of 0.6, 1, 2, 5, 10, 20, 50, and 100 mg/L were freshly prepared by transferring an exact volume

of 600, 1000, 2000 µL of 10 mg/L NH_2OH and 50, 100, 200, 500, 1000 µL of 1000 mg/L, respectively, into 10.00 mL volumetric flask. After that, 100 µL of 1000 mg/L of pyridine was added into the flask to perform as 'a marker' for HPLC system. The volume was adjusted with the mobile phase.

4.4.1.4 Pyridine solution, 1000 mg/L

101.8 µL of pyridine was transferred into 100.00 mL volumetric flask and adjusted to volume using deionized (DI) water.

4.4.1.5 Ammonium acetate buffer pH 5.0

Ammonium acetate buffer pH 5.0 (10 mM acetic acid) solution was prepared by dissolving 1.3395 g of ammonium acetate and 1,144 µL of 50% acetic acid in DI water and the volume was adjusted to 1000 mL in a volumetric flask.

4.4.1.6 Mobile phase

Mobile phase for cation-exchange method consisted of 60:40 (%v/v) Ammonium acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile. The prepared mobile phase was filtrated through nylon membrane filter, 0.45 µm and degassed in ultrasonic bath for 15 minutes.

4.4.2 Solutions for HPLC reversed-phase

4.4.2.1 Stock NH_2OH standard solution, 1000 mg/L

0.0621 g of hydroxylamine sulfate was weighed and then dissolved in DI water. The solution was transferfed into a 25.00 mL volumetric flask and made up to volume with DI water.

4.4.2.2 NH_2OH intermediate standard solution, 10 mg/L

The NH_2OH intermediate standard at 10 mg/L was prepared by transferring 100.0 µL of 1000 mg/L stock NH_2OH standard solution into a volumetric flask 10.00 mL and then adjusted to volume with DI water.

4.4.2.3 Acetone oxime preparation

Acetone oxime was prepared at different concentrations of NH_2OH, i.e., 0.5, 1, 2, 5, 10, 20, and 50 mg/L by transferring the exact volume of 50, 100, 200, 500 µL of 10 mg/L NH_2OH and 10, 20, 50 µL of 1000 mg/L NH_2OH into microtube, respectively. Then, the excessive volume of acetone (5 µL) was added into the microtube. The mixture solution was adjusted to 1.00 mL with DI water and mixed thoroughly using agitator vortex. The procedure of adding solution is summarized in the Table 4.3 below.

Table 4.3 The procedure of adding solution for acetone oxime formation.

Concentration of NH_2OH (mg/L)	NH_2OH (µL)		Acetone (µL)	DI water (µL)
	Stock 10 mg/L	Stock 1000 mg/L		
0.5	50	-	5	945
1	100	-	5	895
2	200	-	5	795
5	500	-	5	495
10	-	10	5	985
20	-	20	5	975
50	-	50	5	945

4.4.2.4 Mobile phase

The mobile phase used for reversed-phase method was 10:90 (%v/v) methanol:DI water. The mobile phase was filtrated through nylon membrane filter, 0.45 µm and degassed in ultrasonic bath for 15 minutes.

4.4.3 Solutions for colorimetric method

4.4.3.1 Stock NH_2OH standard solution, 1000 and 10 mg/L

The preparation of stock NH_2OH standard solution at 1000 mg/L and 10 mg/L were followed up section 4.4.2.1 and 4.4.2.2, respectively.

4.4.3.2 Ferric iron, 2×10^{-3} M

The solution was prepared by dissolving 0.0965 g of ammonium iron (III) sulphate ($FeNH_4(SO_4)_2$) in DI water and added 0.8 mL of 70% nitric acid. The solution was adjusted to 100 mL with DI water in a volumetric flask.

4.4.3.3 Ferrous iron, 1×10^{-3} M

0.0392 g of ammonium iron (II) sulfate ($(NH_4)_2Fe(SO_4)_2$) was dissolved in DI water and adjusted to 100 mL in a volumetric flask. The concentration 1×10^{-3} M of Fe^{2+} was used for studying the Job's plot method.

4.4.3.4 Acetate buffer, pH 5 (500 mM acetic acid)

7.1276 g of sodium acetate and 5.75 mL 50% acetic acid were dissolved together in DI water and the volume was made up to 100 mL in a volumetric flask.

4.4.3.5 *o*-phenanthroline 1×10^{-3} M

1×10^{-3} M *o*-phenanthroline solution was prepared by dissolving 0.1982 g of *o*-phenanthroline in 1000 mL of water.

4.4.3.6 Fe(II)-*o*-phenanthroline complex preparation

The colored complexs of Fe(II)-*o*-phenanthroline were prepared from different concentrations of NH_2OH. First, 1 mL of 2×10^{-3} M Fe^{3+} solution and 500 µL of acetate buffer pH 5 was added into 10.00 mL volumetric flask followed by 2.5 mL of 1×10^{-3} M *o*-phenanthroline solution. Then the exact volume of 50, 100, 200, 500, 1000, 2000 µL of 10 mg/L NH_2OH were added into the flask and

made up to volume with DI water. Blank was the solution that did not add NH_2OH to the flask.

4.4.4 Sulfuric acid, 1.0 M

1.0 M sulfuric acid solution was prepared by dissolving 13.74 mL of 97% sulfuric acid in DI water and adjusting volume to 250 mL in a volumetric flask.

4.5 Procedures

This section describes the procedure in more detail of the developed methods.

4.5.1 Method optimization

Method optimization was carried out by varying the affecting parameters of the analysis. In each the developed method, the variable parameters were difference and described in each following method.

4.5.1.1 HPLC with cation-exchange column

The HPLC method using cation-exchange column was optimized by varying the mobile phase conditions, such as, volume of organic fraction, type of acetate salts, concentration of buffer and pH. The chromatographic separation was evaluated by using NH_2OH and pyridine standard. Pyridine was used as a ‘marker’ for testing cation-exchange process and equilibrium of the column. The column used was Synchrom S300 (250 mm × 4.6 mm) packe with polymeric, hydrophilic, silica-based strong cation exchange support. Prior to analysis, the column was equilibrated with the mobile phase at 10 column volumes and after the final run, the column was cleaned with DI water for 1 hr and kept in 10:90 (%v/v) methanol: DI water.

The optimum HPLC conditions obtained from the optimization process is shown as follow;

Mobile phase	:	60:40 (%v/v) ammonium acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile
Column	:	Synchrom S300 column (250 mm × 4.6 mm)
Flow rate	:	1.0 mL/min
Injection volume	:	10 µL
Detection	:	UV detector at 210 nm

4.5.1.2 HPLC with reversed-phase column

The analysis of NH_2OH on reversed-phase column was carried out by measuring acetone oxime that obtained from the derivatization between NH_2OH and acetone. The formation of acetone oxime was studied by varying reaction time and reaction temperature. The chromatographic parameters affecting the separation of acetone oxime were studied, such as, column type and mobile phase composition. The column used was LiChroCART® (250 mm × 4 mm, RP-18 (5 µm)), C18 column. Before the analysis, C18 column was equilibrated by flowing the mobile phase of 3 to 4 column volumes. After work, the column was cleaned with the mobile phase and then followed by the stronger solvents, e.g., methanol or acetonitrile.

The optimum HPLC conditions for separating and analyzing acetone oxime is shown below;

Mobile phase	:	10:90 (%v/v) methanol: DI water
Column	:	LiChroCART® (250 mm × 4 mm, RP-18 (5 µm))
Flow rate	:	1.0 mL/min
Injection volume	:	10 µL
Detection	:	UV detector at 230 nm

4.5.1.3 Colorimetric method

The colorimetric method using Fe(II)-*o*-phenanthroline complex formation was developed by studying the reaction parameters, such as, effect of pH, reducing power of NH_2OH, kinetic of the reaction, and mole fraction of Fe^{2+}:*o*-phenanthroline.

4.5.2 Rubber extraction method

NH_2OH were extracted from rubber samples by using two extraction method, i.e., solid-liquid extraction and reflux.

4.5.2.1 Solid-liquid extraction

Rubber sample was cut into small pieces with scissors. 1 g of rubber was weighed and placed in a 250 mL Erlenmeyer flask. 50 mL of dichloromethane was added to swell the rubber. A flask was sonicated for 1 hour and then shaked for 5 hours. 20 mL of extraction solvent was added into the flask to extract NH_2OH from organic/rubber phase. The extraction solvent used were ammonium acetate buffer (10 mM acetic acid), pH 5 (*for HPLC cation-exchange analysis*) and DI water (*for HPLC reversed-phase and colorimetry*). The solution was shaken for 1 hour. The flask was placed until aqueous phase separate from organic/rubber phase. The extracted solution were differently prepared for NH_2OH analysis using three developed methods as described below.

4.5.2.1.1 HPLC with cation-exchange

The extracted solution was taken for 6.0 mL and added 4.0 mL of acetonitrile in order to keep NH_2OH in the mobile phase condition. The mixing solution was injected into HPLC with cation exchange column.

4.5.2.1.2 HPLC with reversed-phase

The extracted solution was taken for 500 µL and added 5 µL of acetone to form acetone oxime product. The mixture solution was adjusted to 1 mL with DI water and then mixed thoroughly before reversed-phase HPLC injection.

4.5.2.1.3 Colorimetric method

The extracted solution was taken for 250 µL and then mixed with 1 mL of 2×10^{-3} M Fe^{3+} solution and 500 µL of acetate buffer in a 10.00 mL volumetric flask. Then, 2.5 mL of 1×10^{-3} M *o*-phenanthroline solution was added into the mixture solution to form the complex and adjusted the final volume to 10 mL. The absorbance of the solution was measured using spectrophotometer.

4.5.2.2 Reflux

Rubber sample was cut into small pieces with scissors. 3 g of rubber was weighed and placed in a 250 mL round-bottom flask. 27 mL of 1 M H_2SO_4 used as extractant was added into the flask. Rubber sample was boiled at 100 ºC under reflux condition for 24 hr. A condenser was feed with 10 ºC cooling water that was delivered from circulator cool ace. 250 µL of extractant was prepared for colorimetry by following section 4.5.2.1.3.

4.5.3 Method validation

The validation process was performed to confirm the performance characteristics of the developed method for NH_2OH analysis by following AOAC validation method guideline (AOAC, 2013). The studying parameters were specificity, linearity, detection limit (DL), quantitation limit (QL), precision, and recovery.

4.5.3.1 Specificity (selectivity)

Specificity is the ability of the method to distinguish and quantify the target analyte in the presence of other substances or interference matrices. The parameter used in this study was the resolution of the desired peak from component peaks. Resolution, R_s, is expressed as a function of the separation distance of retention times, t_1 and t_2, and the peak widths, W_1 and W_2 of the analyte peak and nearest peak.

$$R_s = 2\,(t_2-t_1)/(W_1+W_2)$$

4.5.3.2 Linearity

The calibration procedure in this study was performed by using external calibration curve, approximately six to eight points covering the reasonable concentration range. Linear range, regression equation and linearity (r^2) of each developed method were reported.

For HPLC cation-exchange, NH_2OH standard at the concentration of 0.6, 1, 2, 5, 10, 20, 50, and 100 mg/L were prepared in the mobile

phase. The average peak area from triplicate analyses using HPLC cation exchange column was plotted with NH_2OH standard concentration.

For HPLC reversed-phase, acetone oxime derivatives prepared from different concentrations of NH_2OH; 0.5, 1, 2, 5, 10, 20, and 50 mg/L were analyzed in triplicate by HPLC using C18 column. Calibration curve was constructed between the average peak area of acetone oxime and NH_2OH concentration.

For colorimetry, NH_2OH was indirect analyzed by using the Fe(II)-*o*-phenanthroline complex formation. A six-point calibration curve was plotted between the absorbance of the complex (without repeating) and NH_2OH standard concentration at 0.05, 0.1, 0.2, 0.5, 1, and 2 mg/L.

4.5.3.3 Detection limit (DL) and quantitation limit (QL)

The calculation of DL and predicted QL was based on the variability of blank analysis. DL and predicted QL were taken from 3 times and 10 times the standard deviation of blank solution (n = 10); DL = $3SD_{Blank}$ and QL = $10SD_{Blank}$. The predicted QL was reanalyzed with the same method to confirm accuracy and precision of the measurement. (Appendix M)

4.5.3.4 Accuracy and precision

Accuracy and precision of the method were investigated at three concentration levels of NH_2OH standard (low, middle, high) which were different in each method; 0.6, 10, 100 mg/L (for HPLC cation-exchange); 0.5, 5, 50 mg/L (for HPLC reversed-phase) and 0.05, 0.1, 2 mg/L (for colorimetry). The preparation of NH_2OH standard was performed by spiking NH_2OH into aqueous system to imitate the system of the extracted solution. Then the solutions were analyzed with three developed methods using 10 replications. Accuracy and precision were evaluated by using the percentage recovery and relative standard deviation (RSD).

The percentage recovery was calculated by using the ratio of the experimental concentration and the calculated concentration as shown in the equation.

$$\%\text{Recovery (for } NH_2OH \text{ std.)} = \frac{\text{Experimental concentration}}{\text{Actual or calculated concentration}} \times 100$$

The repeatability precision of the method was estimated by using HORRAT equation that is expressed as a ratio of the found value of RSD_r to that calculated from the formula; RSD_r, % = $2C^{-0.15}$ (C expressed as a mass fraction). The acceptable values for the repeatability are 0.3 to 1.3. (Appendix N)

$$HORRAT_r = \frac{RSD_r \text{ (found, \%)}}{RSD_r \text{ (calculated,\%)}}$$

*Intra-day precision was performed the analysis within one day

**Inter-day precision was performed at the same concentration (with intra-day) in three analysis days.

CHAPTER V
RESULTS AND DISCUSSION

This chapter presents the results from the analysis of hydroxylamine (NH_2OH) using three developed methods, i.e., high performance liquid chromatography (HPLC) using cation-exchange column (section 5.1), HPLC using reversed-phase column (section 5.2), and colorimetry technique (section 5.3). These developed methods were applied for the determination of NH_2OH in rubber samples obtained from Michelin company and RTEC by using 2 extraction methods, i.e., solid-liquid extraction and reflux.

5.1 HPLC with cation-exchange column

The column used in this section was a cation-exchange column for separating NH_2OH in positive form, i.e., hydroxylammonium ion (NH_3OH^+). Hydroxylammonium ion can be prepared by treating NH_2OH in acid condition at pH lower than 5.97 (pKa). The separation was achieved by using the ionic equilibrium between the stationary phase ($-SO_3^-$) and the mobile phase (H^+) with the analyte (NH_3OH^+).

The optimization of the method was performed by varying parameters of the mobile phase that was suitable for the UV-Vis detection system. HPLC conditions were operated on Waters LC Module I plus. The column used was Synchrom S300 column (250 mm × 4.6 mm) with the flow rate of the mobile phase at 1.0 mL/min.

5.1.1 Chromatographic optimization

The optimization process was performed by using NH_2OH standard and used pyridine for checking the cation-exchange efficiency.

As described above, NH_2OH must be treated under acidic condition at pH lower than 5.97 to form NH_3OH^+. Therefore, the acidic mobile phases, i.e., 10 mM

nitric acid, 10 mM acetic acid, 10 mM sulfuric acid, 10 mM phosphoric acid, and 60:40 (%v/v) ammonium acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile were used for determining NH_2OH (Appendix B). The preliminary results showed that the mobile phase of a mixture of acetate buffer pH 4.5:acetonitrile at the ratio of 60:40 (%v/v) was suitable for the study.

The absorption spectrum of NH_2OH in the mobile phase consisted of 60:40 (%v/v) ammonium acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile was investigated on a spectrophotometer using scan mode from 200-600 nm (Figure C.1). The maximum absorbance was shown at 210 nm within the absorption range of 200-250 nm. A small molecule without chromophore such as NH_2OH can absorb UV light because of the phenomenon like “indirect absorbance detection” that NH_2OH change the absorbance of the mobile phase.

The following sections present the optimization of the mobile phase composition, such as, volume of organic fraction, type of acetate salts, concentrations of buffer and pH of buffer in the mobile phase.

5.1.1.1 Volume of organic fraction

Volume of acetonitrile in the mobile phase consisted of ammonium acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile was varied from 10-40 (%v/v). The chromatograms are shown in Figure 5.1. The proportion between acetonitrile and acetate buffer in the mobile phase affected the retention time of both pyridine and NH_2OH and also affected the sensitivity of mostly NH_2OH signal. Due to pyridine signal comes from UV absorption, while NH_2OH signal comes from indirect absorbance detection, therefore, the decreasing of acetonitrile caused the decreasing of NH_2OH signal.

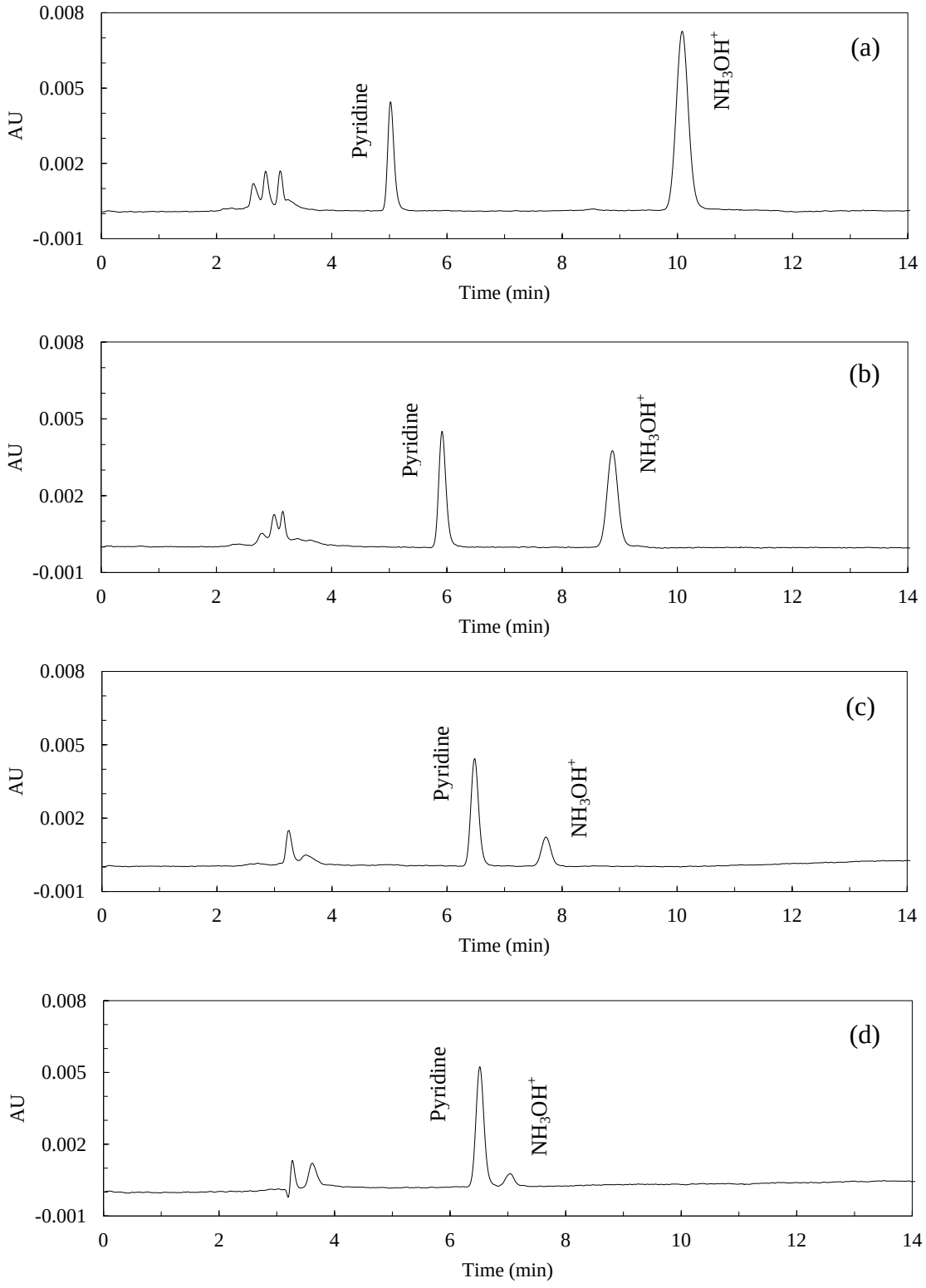


Figure 5.1 HPLC chromatograms of 10 mg/L of pyridine and 20 mg/L of NH_2OH using various ratio of ammonium acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile; (a) 60:40 (%v/v), (b) 70:30 (%v/v), (c) 80:20 (%v/v), and (d) 90:10 (%v/v).

5.1.1.2 Type of acetate salts in the mobile phase

Using different types of buffer salt in the mobile phase can affect the separation efficiency in cation-exchange column because of the diffence in ionic strength of the mobile phase. (Pohl, Stillian, & Jackson, 1997) (Ionic strength is a parameter concerning about total ion concentration and ion charge in the solution). In this work, the mobile phase with the same concentration of acetate buffer (10 mM acetic acid) at pH 5.0 was prepared from sodium acetate salt (CH_3COONa) and ammonium acetate salts (CH_3COONH_4). The chromatograms are shown in Figure 5.2. The mobile phase of CH_3COONa with Na^+ ion has stronger ionic strength than the mobile phase of CH_3COONH_4 with NH_4^+ ion. It is because of the dissociation of the buffer salt in the solution. The dissociation constant (K_a) of CH_3COONa and CH_3COONH_4 were reported at 2.63×10^{-7} M and 1.00×10^{-7} M, respectively (Proll & Sutcliffe, 1961). Higher K_a value indicates the better dissociation ability of the compound in solution. Therefore, at the same concentration of acetate salt, CH_3COONa gives more strength of the mobile phase than CH_3COONH_4.

5.1.1.3 Concentration of buffer

This experiment studied the effect of buffer concentration on the ionic strength of the mobile phase. The acetate buffer solutions containing different concentrations of CH_3COOH and CH_3COONH_4 were prepared by maintaining pH value around 4.5. The proportion of CH_3COOH (mM):CH_3COONH_4 (mM) were studied at the ratio of 5:2.7, 10:5.5, 20:11, and 30:16.5. Higher concentration of NH_4^+ and H^+ related to higher ionic strength of the mobile phase resulting in faster elution of NH_3OH^+, as shown in Figure 5.3. The retention time of NH_2OH correlated with the highest ionic strength to lowest ionic strength of the mobile phase was 6.9, 7.9, 10.1, and 11.8 minutes, respectively.

5.1.1.4 pH of the mobile phase

Effects of pH of the mobile phase are shown in Figure 5.4. The mobile phase consisted of 60% acetate buffer varying pH from pH 4.0 – 5.5 with the same concentration of acetic acid at 10 mM and 40% acetonitrile. To obtain the required pH, the concentration of CH_3COONH_4 was changed. The proportion of

CH_3COOH (mM):CH_3COONH_4 (mM) were 10:1.7 (pH 4.0), 10:5.5 (pH 4.5), 10:17 (pH 5.0), and 10:55 (pH 5.5). Higher pH value gave more elution strength of the mobile phase because of higher concentration of NH_4^+ ion. The pH value of the solution affect on the sensitivity of NH_2OH signal. However, pH value at 5.0 was selected because this condition showed better retention time and higher sensitivity.

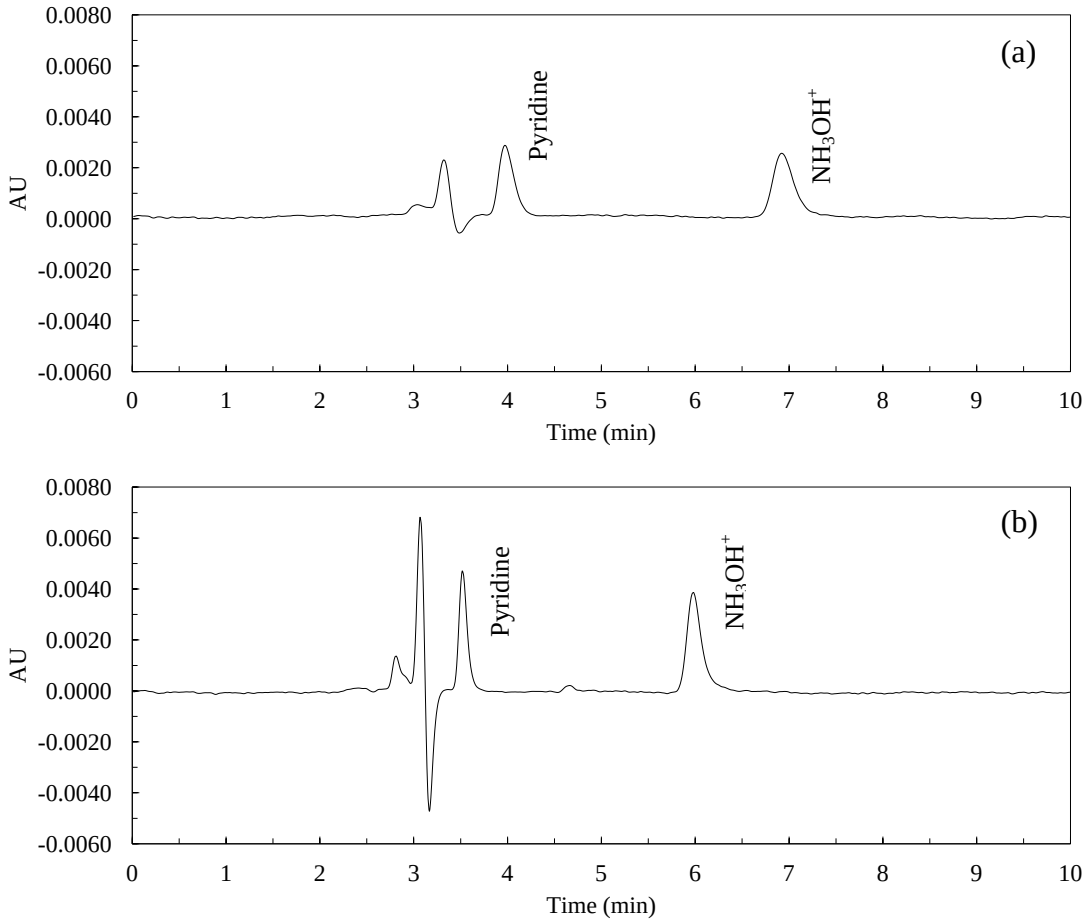


Figure 5.2 HPLC Chromatogram of 10 mg/L of pyridine and 20 mg/L of NH_2OH using 60:40 (%v/v) acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile that was prepared from different types of acetate salt; (a) ammonium acetate salt and (b) sodium acetate salt.

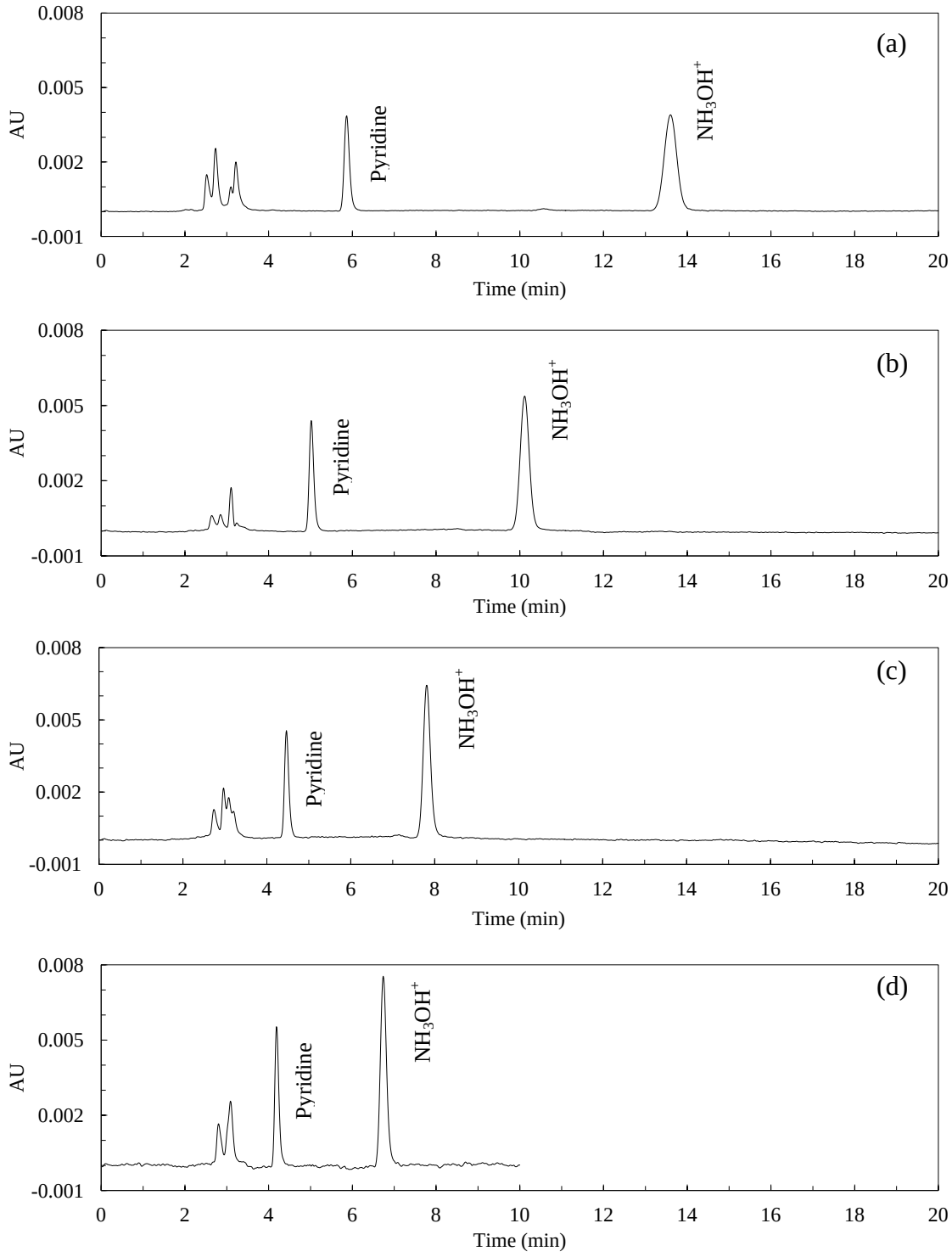


Figure 5.3 HPLC chromatograms of 10 mg/L of pyridine and 20 mg/L of NH_2OH using 60:40 (%v/v) ammonium acetate buffer pH 4.5:acetonitrile as the mobile phase, varying the concentration of CH_3COOH (mM):CH_3COONH_4 (mM); (a) 5:2.7, (b) 10:5.5, (c) 20:11, and (d) 30:16.5.

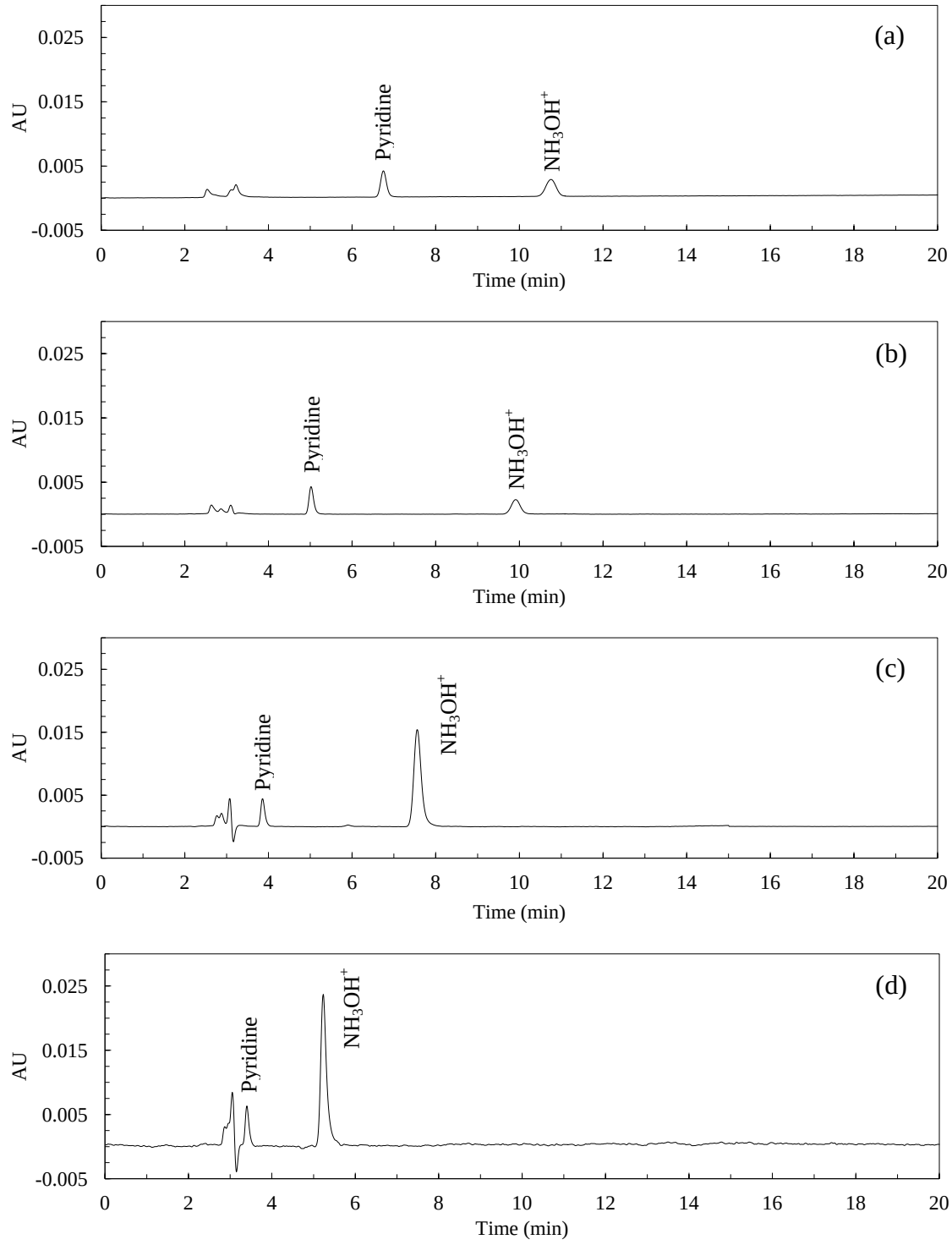


Figure 5.4 HPLC chromatograms of 10 mg/L of pyridine and 20 mg/L of NH_2OH using 60:40 (%v/v) ammonium acetate buffer (10 mM acetic acid) with various pH values:acetonitrile; (a) pH 4.0, (b) pH 4.5, (c) pH 5.0, and (d) pH 5.5.

5.1.2 Analytical performances

The optimum condition of HPLC cation-exchange was achieved on a strong cation-exchange column (Synchrom S300 column 250 mm × 4.6 mm) using isocratic elution of 60:40 (%v/v) ammonium acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile. The flow rate was used at 1.0 mL/min, the injection volume was 10 µL and the UV-Vis detector was set at 210 nm.

The performance of the HPLC cation-exchange method was evaluated by following the AOAC validation method guideline (AOAC, 2013). The tested performance characteristics were specificity, linearity, detection limit, quantitation limit, precision, and recovery.

Specificity was observed from the HPLC chromatogram using the optimum condition, as shown in Figure 5.4 (c). The retention time of pyridine and NH_3OH^+ was 3.9 and 7.6 minutes, respectively. Resolution, $R_s = 2\ (t_2 - t_1)/(W_1 + W_2)$ between pyridine peak and NH_3OH^+ peak showed the value of 4.3 (higher than acceptable value of 1.5).

Calibration curve of NH_2OH standard was constructed at eight consecutive concentrations at 0.6, 1, 2, 5, 10, 20, 50, and 100 mg/L with three replicated of the analyses. The linear regression line is shown in Figure 5.5.

Precision of the method was evaluated in term of intra-day and inter-day precision by using percentage of relative standard deviation (%RSD) and HORRAT equation. Recovery value indicates the accuracy of the method by comparing between experimental value and actual value (calculated value or certified value) and also indicates the repeatability of the method.

All of the analytical performances described above are shown in Table 5.1 and Table 5.2.

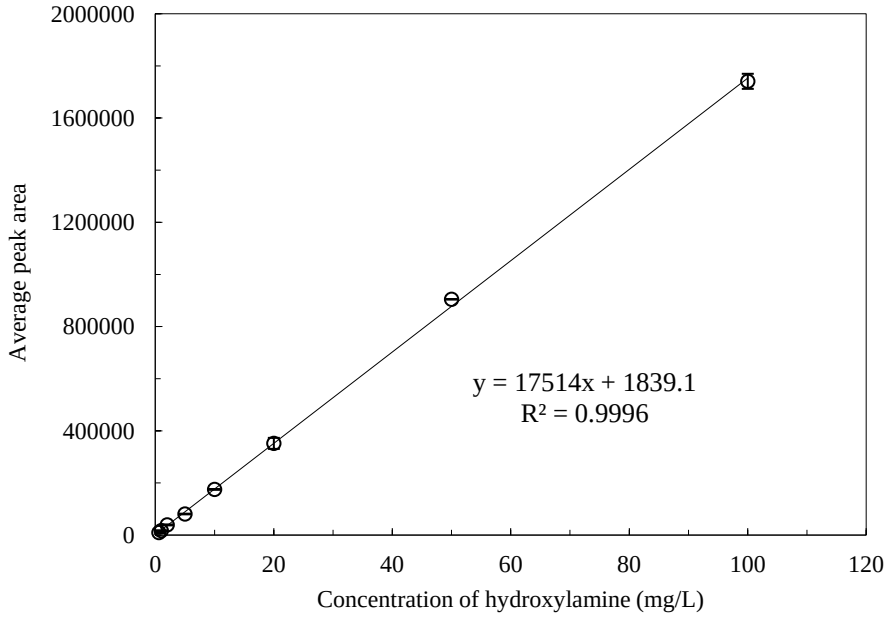


Figure 5.5 Calibration curve of standard NH_2OH for the HPLC cation-exchange method.

Table 5.1 Analytical data of the HPLC cation-exchange method for standard NH_2OH under the optimum conditions.

Linear range (mg/L)	**Regression equation**	**Linearity (r^2)**	**DL (mg/L)**	**QL (mg/L)**
0.6 - 100	y = 17514x + 1839.1	0.9996	0.35	0.54

Table 5.2 Method precision and recovery for chromatographic analysis of standard NH_2OH added into aqueous system (n = 10).

	0.6 mg/L		**10 mg/L**		**100 mg/L**	
	%RSD	**%Recovery**	**%RSD**	**%Recovery**	**%RSD**	**%Recovery**
Day 1	4.2	118.9	2.7	151.5	5.4	131.0
Day 2	6.4	76.2	2.7	138.8	2.6	136.6
Day 3	8.3	86.9	3.4	139.5	3.2	134.8
Inter-day	23.6	94.0	5.0	143.3	2.1	134.1

The calculation for precision and recovery was shown in Appendix N. The method showed that the precision at three concentrations presented in the accetable range of AOAC, but, the method showed unacceptable recovery value at the concentration higher than 10 mg/L which were in the range of 134.1-143.3% (the acceptable range was 75-120%). The signal of NH_2OH standard in 3 consecutive measurements showed significantly increasing with time. Therefore, the repeatability of the NH_2OH at the same concentration was not acceptable and then the experiment for the repeatability studied was designed by measuring the same solution of NH_2OH 10 mg/L at different time from 1 to 9 hours after preparing the solution. The results showed significantly increasing of the signal of NH_2OH according the increasing time, as shown in Figure 5.6.

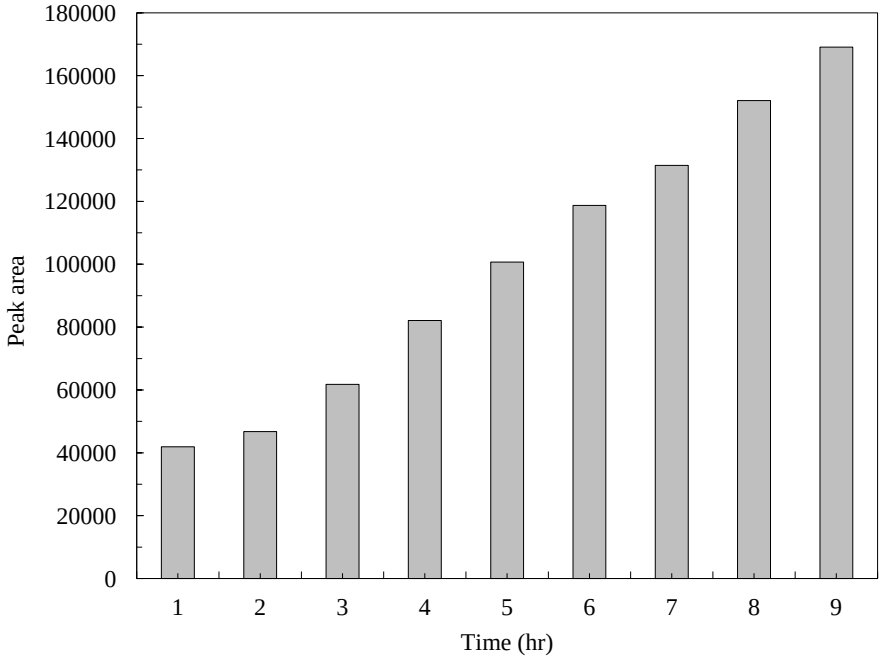


Figure 5.6 The increase of peak area with time from measuring the same solution of 10 mg/L NH_2OH.

The results showed that the retention time at 7.6 minutes was not belong to NH_2OH in the analysis form of NH_3OH^+, but NH_2OH in this HPLC system would change to be undesired product from the reaction between NH_2OH and the component

in the mobile phase. This product was more sensitive for UV absorption than NH_3OH^+ and the absorption occurred higher when kept in a longer time before the injection.

Therefore, the experiment to examine the reaction occurring in the mobile phase was designed by comparing NH_2OH in DI water and in the mobile phase. The solutions were stored for daily check within 3 days by using the complex formation of Fe(II)-*o*-phenanthroline. The absorbance of the complexes was measured and plotted the correlation with solution storage time (day). The result is shown in Figure 5.7. The absorbance of the complex prepared from NH_2OH in DI water showed insignificant difference with the storage time in 3 days. However, the solution of NH_2OH prepared in the mobile phase showed significant decrease in the absorbance value within 3 consecutive days of the measurement. The results indicated that the composition in the mobile phase consisted of acetate buffer and acetonitrile reacted with NH_2OH and form other products.

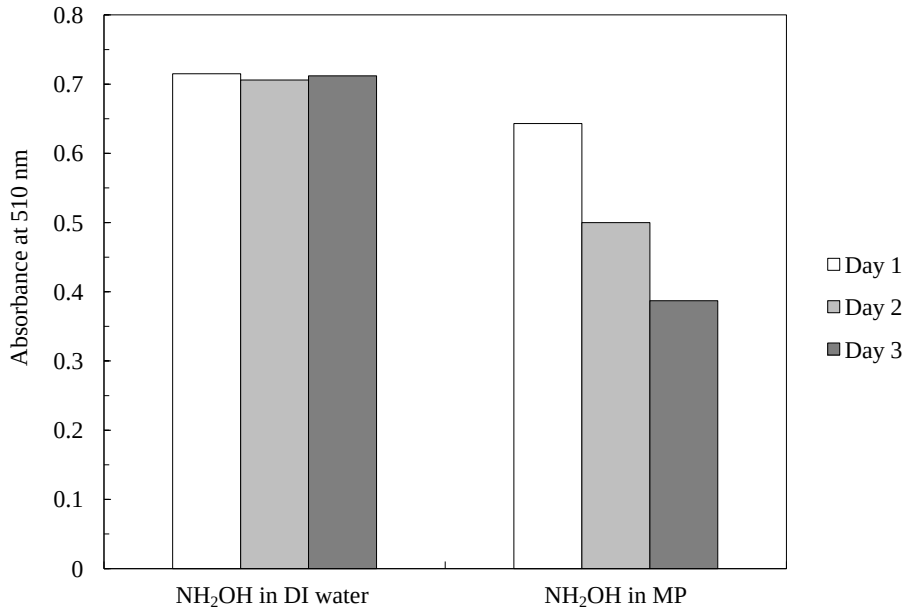


Figure 5.7 3-Days monitoring of the quantity of NH_2OH dissolved in mediums of water and mobile phase.

Based on the literature, NH_2OH can react with acetonitrile to form a product that can absorb UV-Vis light (Brown, Cansfield, Congreve, PICKWORTH, &

TEHAN, 2014), as shown in Figure 5.8. The obtained product will be N'-hydroxyacetimidamide or acetamide oxime.

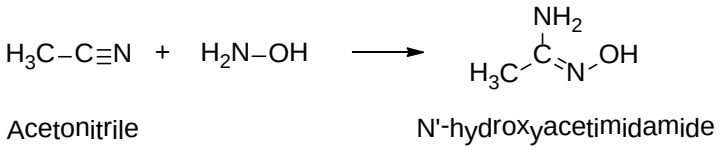


Figure 5.8 The reaction schemes of NH_2OH and acetonitrile.

The variation of organic solvent in the mobile phase between acetonitrile and methanol was performed to confirm the proposed reaction. The experiment was designed by using the same proportion of mobile phase containing 60:40 (%v/v) of acetate buffer, pH 4.5 and organic solvent that varying between acetonitrile and methanol. The chromatograms are shown in Figure 5.9. The signal of NH_2OH was not observed in HPLC chromatogram using methanol as the mobile phase. It can be concluded that the product at t_R = 7.6 minutes was from the reaction between NH_2OH and acetonitrile.

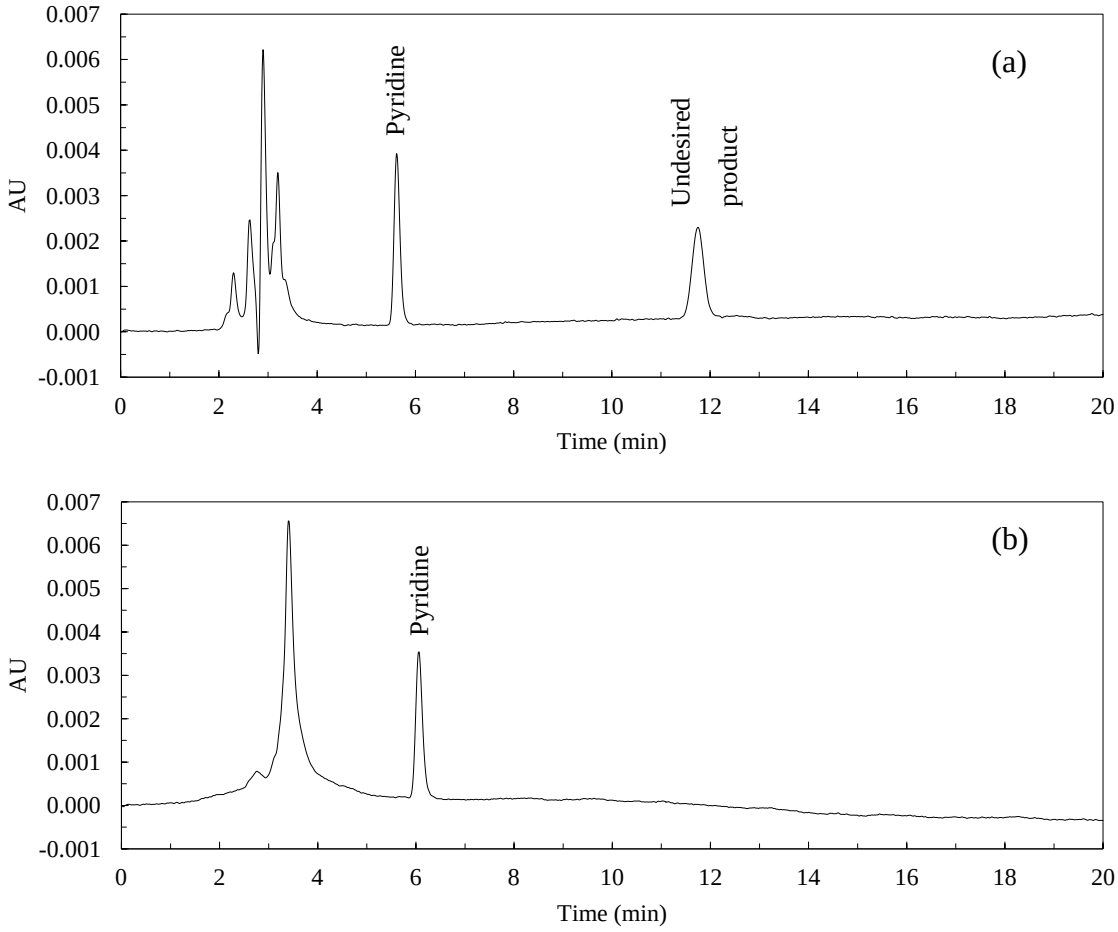


Figure 5.9 HPLC chromatograms of 10 mg/L of pyridine and 30 mg/L of NH_2OH using different organic solvent; (a) 60:40 (%v/v) acetate buffer pH 4.5:acetonitrile, and (b) 60:40 (%v/v) acetate buffer pH 4.5:methanol.

Therefore, using HPLC cation-exchange column with the mobile phase of 60:40 (%v/v) acetate buffer:acetonitrile was not suitable for NH_2OH analysis because the undesired reaction between NH_2OH and acetonitrile was occurred and the obtained signal increased with time. To use this reaction for NH_2OH determination, the parameters affecting the reaction, such as, reaction time, reaction temperature or pH of solution need to be further studied. The other option is to change the detector used for the HPLC with cation-exchange column, such as, conductometric detector because it can directly detect NH_2OH in the form of NH_3OH^+. However, this method was not further applied for determining NH_2OH in the rubber sample.

5.2 HPLC with reversed-phase column

In this work, we developed the reverse-phase chromatography for analysis of NH_2OH on a non-polar stationary phase column (Octadecyl carbon chain (C18)-bonded silica). However, the molecule of NH_2OH is more polar and has no chromophore, so it will not partition on the non-polar column and cannot absorb UV light at the detector used. In order to use this type of column with UV detector, the derivatization reaction is required for converting NH_2OH to be less polar compound that can absorb UV light at the detector. Acetone oxime reaction is a promising derivatized method for converting NH_2OH (Marasco, 1926). Oximes or $R^1R^2C{=}NOH$ are the derivatives of carbonyl group which is usually generated by the reaction between NH_2OH and aldehydes or ketones (Khatoon, 2014). Acetone oxime can be generated from the reaction between NH_2OH and acetone, as shown in Figure 5.10.

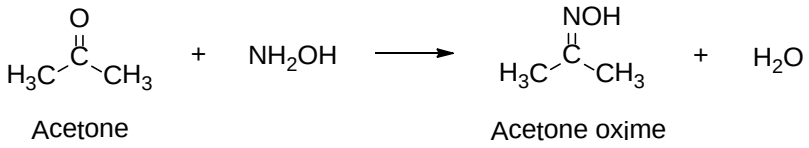


Figure 5.10 The reaction schemes of NH_2OH and acetone.

The reaction of forming acetone oxime from NH_2OH and acetone can be performed under room temperature and the reaction is completely within a few minute. In the study, we carried out the experiment to study effects of reaction temperature and reaction time on the acetone oxime formation. It was observed that the signal respond of the acetone oxime were not significantly changed up to the temperature of 90 ºC and the reaction time at 90 minutes. The results are then shown in Appendix D.

5.2.1 Chromatographic optimization

First, the absorption spectrum of acetone oxime was measured using spectrophotometer and it showed the maximum in the range of 200–260 nm (Figure C.3). The detection at 230 nm was selected for the UV-Vis detector in the HPLC system because of high sensitivity and wide linear range.

For using HPLC reversed-phase with acetone oxime analysis, Li, et al. ((Li, Chen, Jin, & Xing, 2010)) proposed the use of non-polar column partitioning with the polar mobile phase, such as, ZORBAX Eclipse XDB-C18 (4.6 mm × 250 mm, 5 um) flowed with the mobile phase of 25:75 (%v/v) Methanol:DI water. In this study, we developed HPLC analysis of acetone oxime on C18 column with the mobile phase of Methanol:DI water and using UV-Vis detector. The following sections present the optimization of the chromatographic parameter consisted of column type and the mobile phase composition.

5.2.1.1 Column selection

In this study, we carried out on two type of the column (1) Nova-Pak® C18 (150 mm × 3.9 mm) and (2) LichroCART® C18 (250 mm × 4 mm) by using the mobile phase of 25:75 (%v/v) of methanol:DI water. The chromatograms of acetone oxime (50 mg/L of NH_2OH) analyzed by using different types of C18 column are shown in Figure 5.11. The longer column, LichroCART® C18 was selected to use in this work because acetone oxime signal can be clearly separated from acetone peak and was not interfered with other matrices.

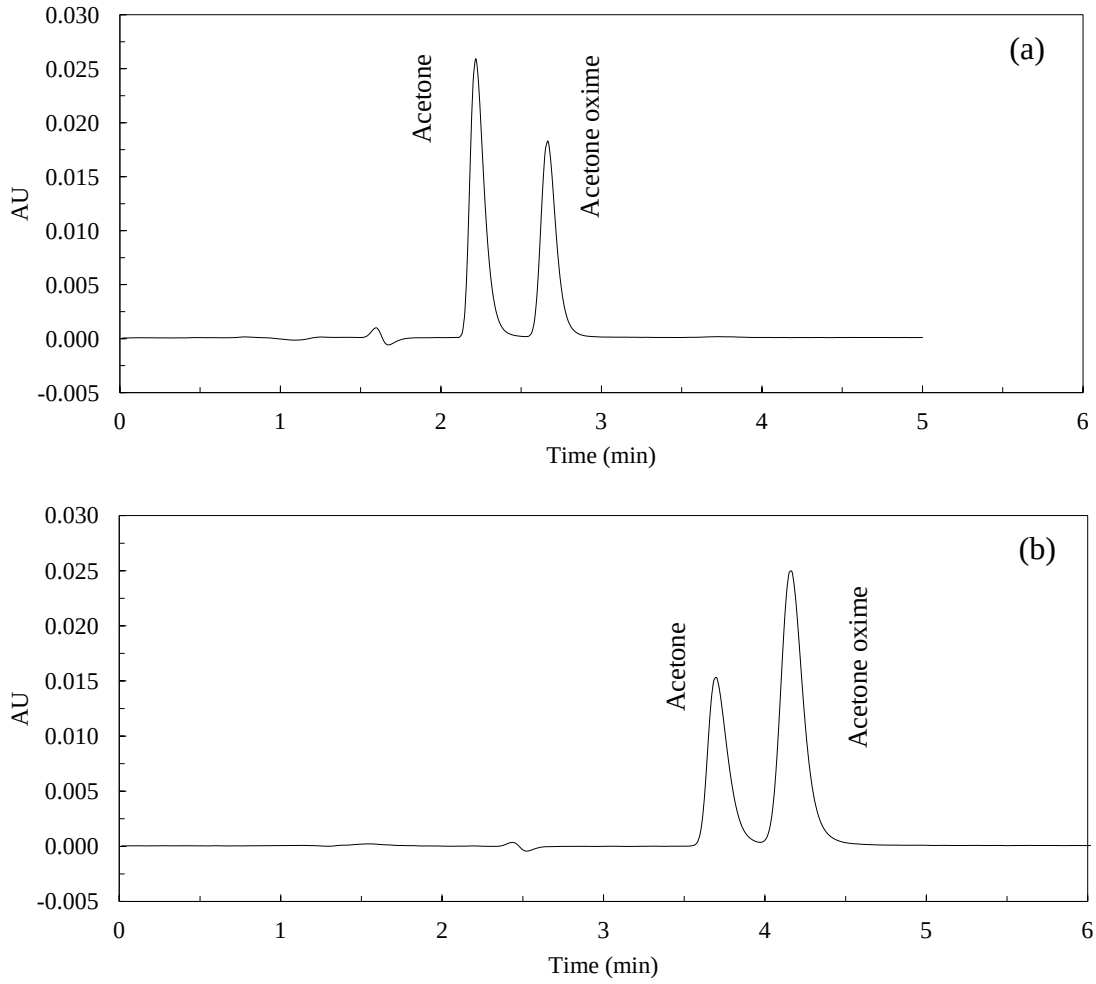


Figure 5.11 HPLC chromatograms of acetone and acetone oxime (50 mg/L of NH_2OH) using different types of column; (a) Nova-Pak® C18 (150 mm × 3.9 mm), and (b) LichroCART® C18 (250 mm × 4 mm).

5.2.1.2 Mobile phase composition

In this section, varying the composition between methanol and DI water of the mobile phase was studied at the ratio of 25:75 (%v/v), 20:80 (%v/v), 15:85 (%v/v), and 10:90 (%v/v). The decreased of methanol ratio reduced the elution strength of the mobile phase which affect the separation of acetone and acetone oxime signal. The chromatograms of each condition are shown in Figure 5.12. The mobile phase ratio of 10:90 (%v/v) methanol:DI water showed the best separation between two peak and also the best peak shape and sensitivity.

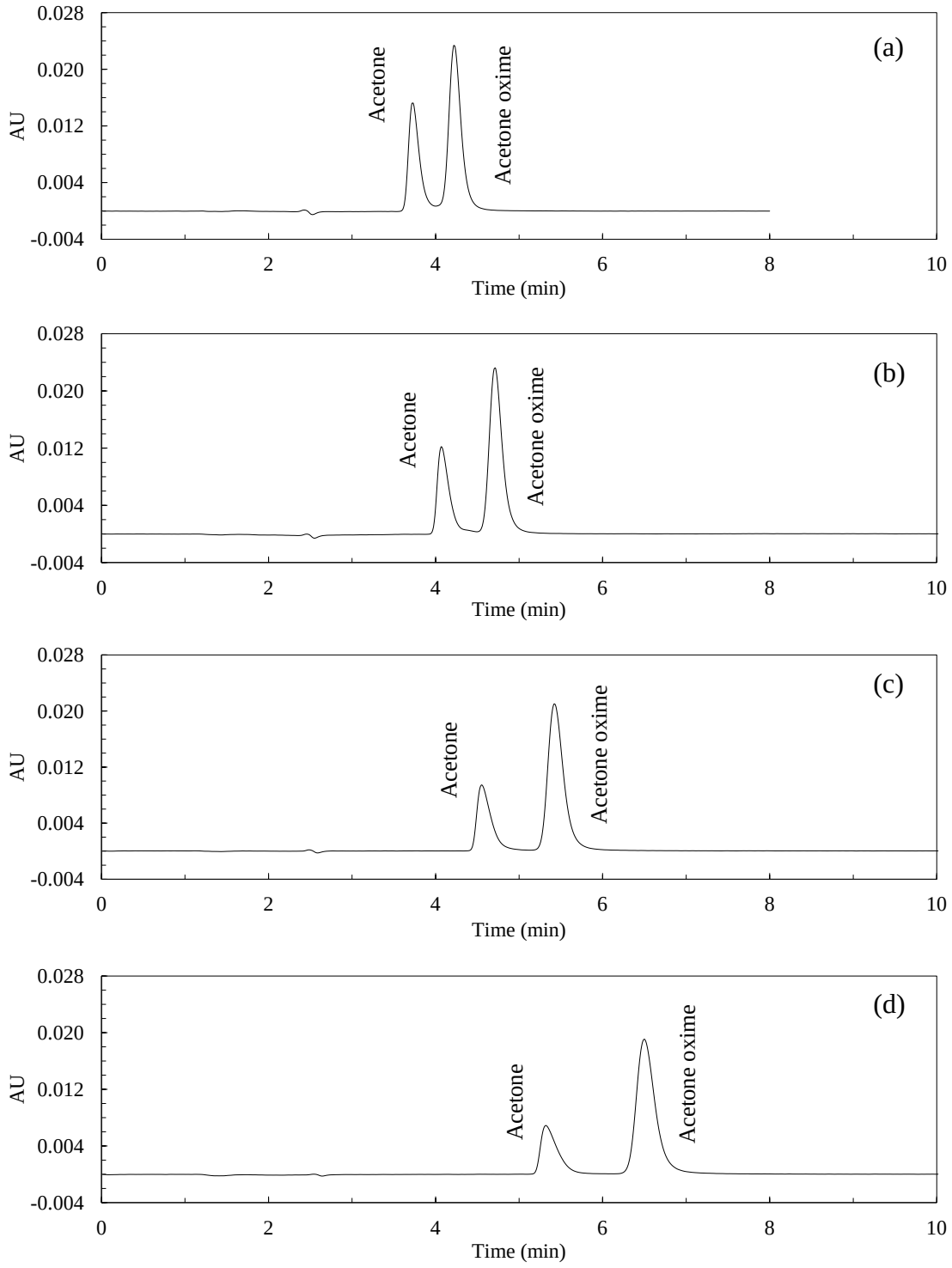


Figure 5.12 HPLC chromatograms of acetone and acetone oxime (prepared from 50 mg/L of NH_2OH) using different mobile phase proportion; (a) 25:75 (%v/v), (b) 20:80 (%v/v), (c) 15:85 (%v/v), and (d) 10:90 (%v/v) of methanol:DI water.

5.2.2 Analytical performances

The optimum condition of the reverse-phase HPLC was carried out on the C18 column; LichroCART® C18 (250 mm × 4 mm) and the mobile phase used was 10:90 (%v/v) methanol:DI water system with flow rate at 1.0 mL/min. UV detector was used at 230 nm. The evaluation of the analytical performance of the method was followed by AOAC validation guideline (AOAC, 2013).

The retention times of acetone and acetone oxime were 5.3 and 6.5 as shown in the HPLC chromatogram (Figure 5.12(d)). The resolution between acetone and acetone oxime was 1.3 (the minimum usable separation followed by AOAC was 1.0).

Seven-points calibration curve of acetone oxime (Figure 5.13) was constructed in the range of 0.5-50 mg/L correlated to NH_2OH. Three replicated analyses were performed at each concentration. Linearity, DL and QL of this method are shown in Table 5.3. The accuracy and precision of the method were evaluated by using percentage recovery and percentage relative standard deviation (%RSD) measuring at three concentrations of NH_2OH, i.e., 0.5, 5, and 50 mg/L (n=10), as shown in Table 5.4. The method showed the accepatble accuracy and precision followin up AOAC guideline.

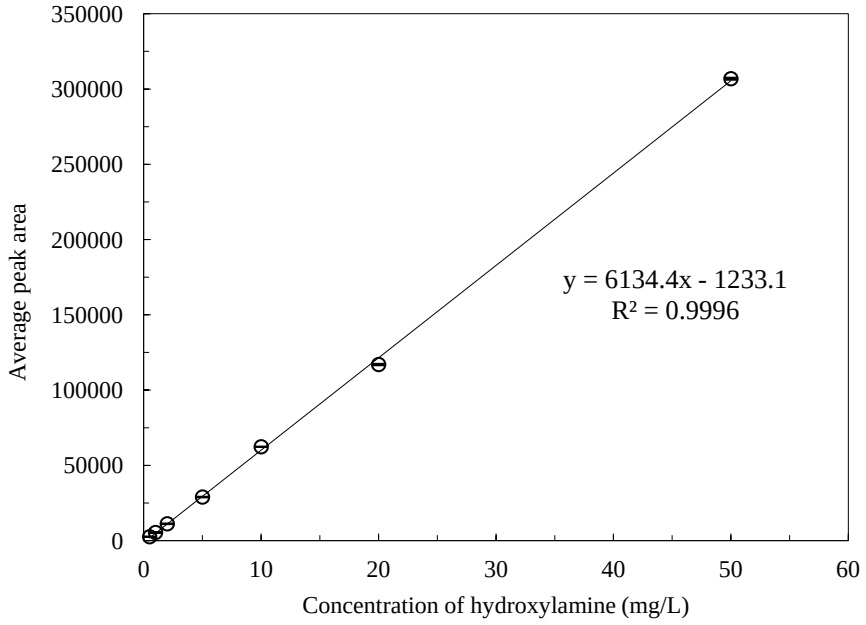


Figure 5.13 Calibration curve of standard NH_2OH for the HPLC reversed-phase method.

Table 5.3 Analytical data of the HPLC reversed-phase method for standard NH_2OH under the optimum conditions.

	Linear range (mg/L)	**Regression equation**	**Linearity (r^2)**	**DL (mg/L)**	**QL (mg/L)**
Acetone oxime (NH_2OH)	0.5 – 50	y = 6134.4x - 1233.1	0.9996	0.16	0.40

Table 5.4 Method precision and recovery for chromatographic analysis of standard NH_2OH added into aqueous system (n = 10).

	0.5 mg/L		**5 mg/L**		**50 mg/L**	
	%RSD	**%Recovery**	**%RSD**	**%Recovery**	**%RSD**	**%Recovery**
Day 1	3.3	135.6	0.5	100.8	5.2	95.4
Day 2	3.9	125.3	0.6	98.0	4.3	96.3
Day 3	4.8	120.2	0.9	98.6	3.8	97.0
Inter-day	6.2	127.0	1.4	99.1	0.9	96.2

5.3 Colorimetric method

In this study, we used the indirect method for analysis of NH_2OH by forming a red color complex of Fe(II)-*o*-phenanthroline that can be absorbed UV-Vis light. NH_2OH (as reducing agent) can reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) and then Fe^{2+} ion will form complex with *o*-phenanthroline, as shown in Figure 5.14. A red stable color complex of Fe(II)-*o*-phenanthroline was measured using UV-visible spectrophotometer.

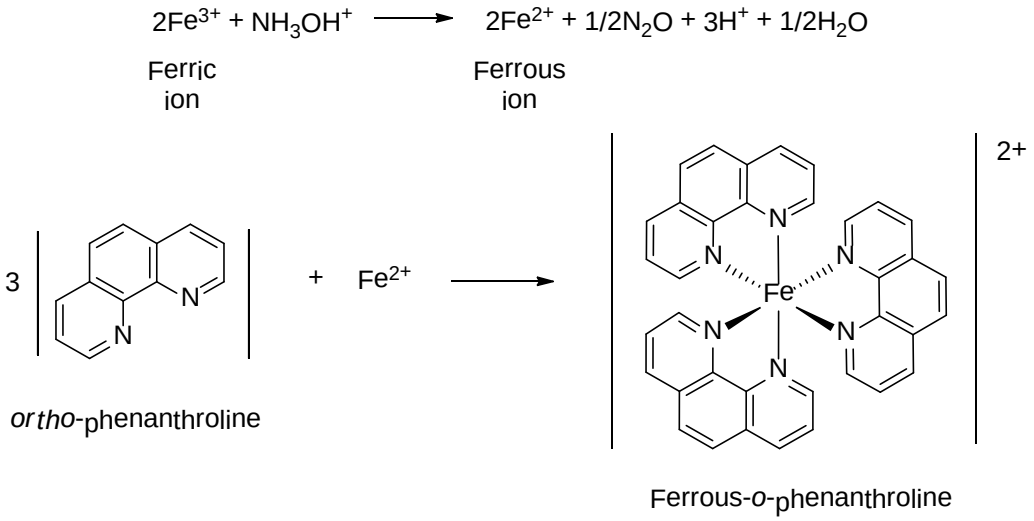


Figure 5.14 The reaction schemes of the formation of Fe(II)-*o*-phenanthroline complex.

In the experiment, the absorption spectrum of Fe(II)-*o*-phenanthroline complex was investigated and then the parameters that affect the complex formation were studied.

5.3.1 Absorption spectrum of Fe(II)-*o*-phenanthroline

Absorption spectrum of Fe(II)-*o*-phenanthroline complex was measured by scanning the wavelength from 200 to 600 nm. The complex showed the high absorbance in range of 350-600 nm and showed the maximum absorption at 510 nm, as shown in Figure 5.15. This wavelength was used for detecting Fe(II)-*o*-phenanthroline complex in this work.

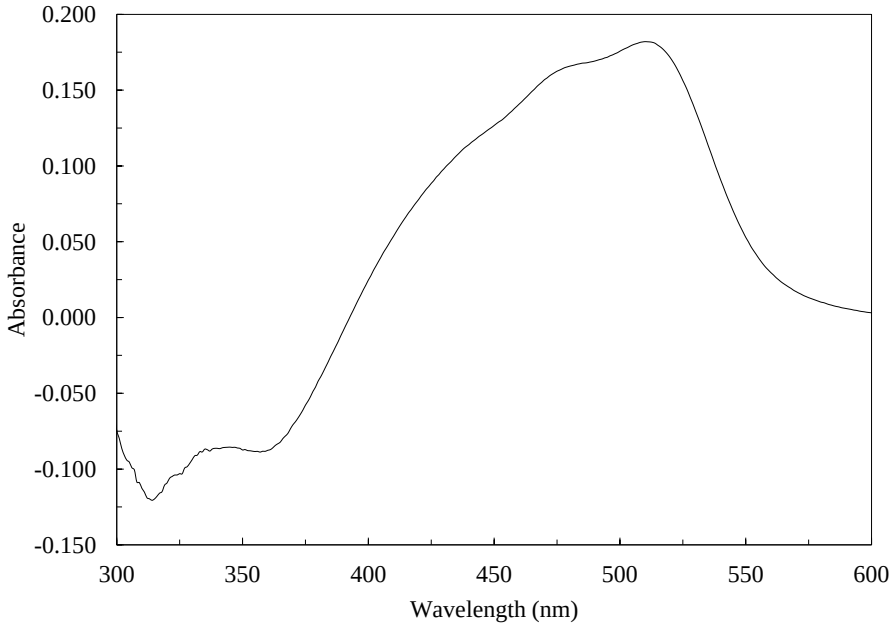


Figure 5.15 Absorption spectrum of Fe(II)-*o*-phenanthroline.

5.3.2 Effect of pH

The pH of the solution is an important parameter in forming Fe(II)-*o*-phenanthroline complex because of the occurrence of the side reactions. At low pH (higher H^+), H^+ ion in the solution will force the protonation of *o*-phenanthroline (1) and at high pH (higher OH^-), the hydroxide forming of Fe^{2+} and F^{3+} ions is occurred (2 and 3).

$$\text{*o*-phenanthroline (aq)} + 2H^+ \text{ (aq)} \rightleftharpoons H_2\text{- *o*-phenanthroline (aq)} \quad (1)$$

$$Fe^{2+} + 2OH^- \text{ (aq)} \rightleftharpoons Fe(OH)_2 \text{ (aq)} \quad (K_{sp}* = 1.6\times10^{-14}) \quad (2)$$

$$Fe(OH)_2 \text{ (aq)} \rightleftharpoons Fe(OH)_3 \text{ (s)} \quad (K_{sp} = 1.1\times10^{-36}) \quad (3)$$

*‘K_{sp}’ or ‘solubility product constant’ is the equilibrium constant indicating the ability of solid substance to dissolve in water. The higher Ksp value present the more soluble of substance.

In the experiment, the pH of the solution were adjusted in the range of 3 to 9 by adding diluted H_2SO_4 or diluted NaOH into Fe^{2+} solutions before forming complex with *o*-phenanthroline. The result is shown in Figure 5.16. It was observed

that at pH 5 was the suitable condition for the reaction as shown the maximum value of the absorbance. Therefore, the acetate buffer pH 5 was used to control pH of the complex solution.

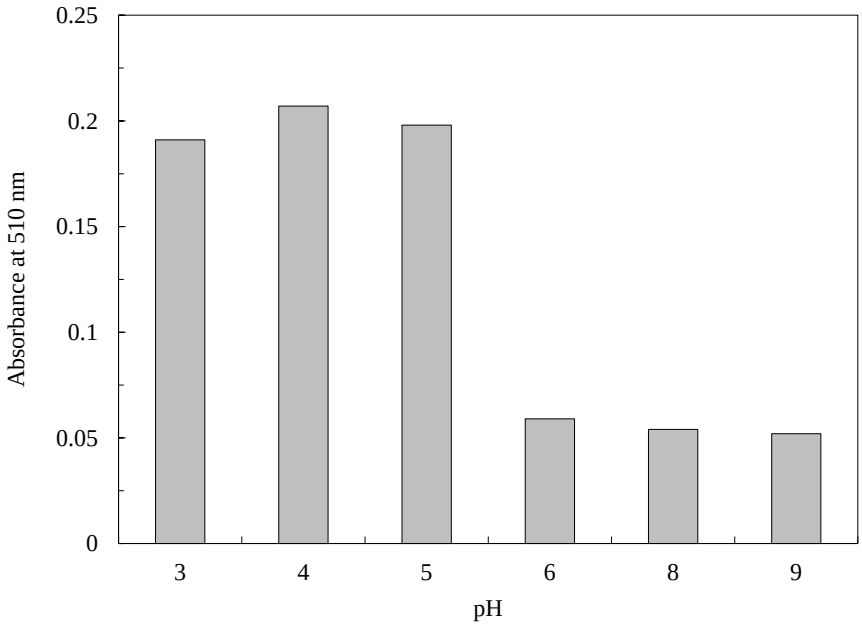


Figure 5.16 Effect of pH on the formation of Fe(II)-*o*-phenanthroline complex.

5.3.3 Kinetic study of the complex

Chemical reaction kinetics is the rate of the reaction processes at which reactants are used and products are formed. Under different reaction conditions, such as, pH or concentration, the reaction will be observed in different kinetic behavior (Adhikamsetty, Gollapalli, & Jonnalagadda, 2008). This section was to study the kinetic of the formation of Fe(II)-*o*-phenanthroline complex at diffent controlling pH value. The experimentation was performed by varying pH of the solution in the range of 4.0–5.5 and then monitoring the absorbance after preparing the solution from t = 0 to t = 3000 seconds (50 minutes) (Figure 5.17). At pH 4.0, the absorbance of complex increased from 0.301 to 0.673 and then became constant at 30 minutes. It showed the slowly formation of the complex that was complete at 30 minutes. At pH 5.0 (the interested pH value), the result showed the insignificantly change of absorbance depend on time (~0.692 to 0.696). It indicated that the complex could be completely generated since t = 0 and would be stable for 50 minutes.

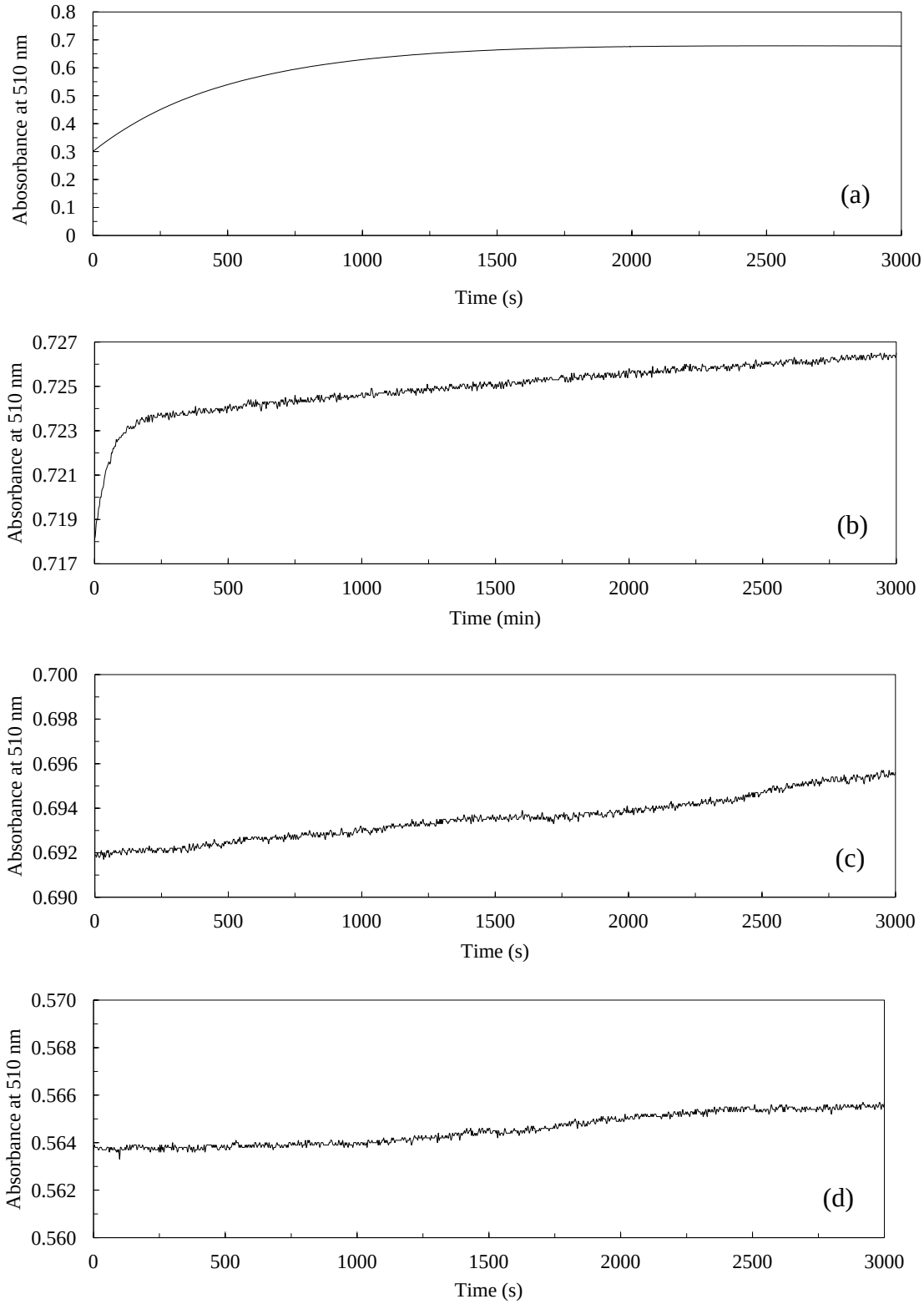


Figure 5.17 The kinetic study of Fe(II)-*o*-phenanthroline complex formation at different pH; (a) 4.0, (b) 4.5, (c) 5.0, and (d) 5.5.

5.3.4 Reducing power of NH_2OH

The reducing power of NH_2OH to reduce Fe^{3+} to Fe^{2+} were studied by varying the concentrations of Fe^{3+} from 2.5×10^{-6} to 30×10^{-6} M to react with the fixed concentration of 5×10^{-6} M NH_2OH. The absorbance of each condition was plotted, as shown in Figure 5.18. The absorbance increased with the increasing of Fe^{3+} concentration until at 20×10^{-6} M that the absorbance become constant. It indicated that 1 mole of NH_2OH can reduce 4 mole of Fe^{3+} or reducing ratio at 4:1 of Fe^{3+}:NH_2OH.

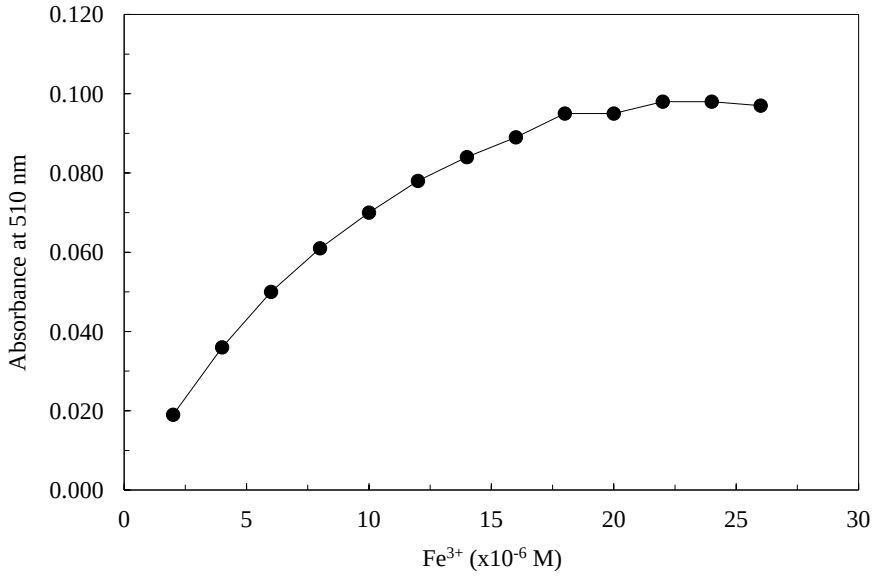


Figure 5.18 Reducing power of 5×10^{-6} M NH_2OH for the reduction of $Fe^{3+} + e^- \rightarrow Fe^{2+}$ by observing the absorbance of Fe(II)-*o*-phenanthroline complex.

5.3.5 Job's plot

In 2008, Adhikamsetty (Adhikamsetty et al., 2008) studied the formation of Fe(II)-*o*-phenanthroline complex and indicated the mole ratio of the complex at 1:3 Fe^{2+}:*o*-phenanthroline. A simplified reaction between Fe^{2+} and *o*-phenanthroline is shown below.

$$xFe^{2+}{}_{(aq)} + y(o\text{-phen})_{(aq)} \rightarrow [Fe_x(o\text{-phen})_y]^{2+}{}_{(aq)}$$

In order to investigate the optimum mole ratio of the complex, the Job's plot method was used. The experiment was performed by varying mole fraction between Fe^{2+} and *o*-phenanthroline at the constant value of total mole number and the result was shown in Figure 5.19. The maximum absorbance was obtained at 0.25:0.75 or 1:3 mole fraction of Fe^{2+}:*o*-phenanthroline indicating the stoichiometric composition of the complex. Therefore, the formula of the complex was $[Fe(o\text{-phen})_3]^{2+}$.

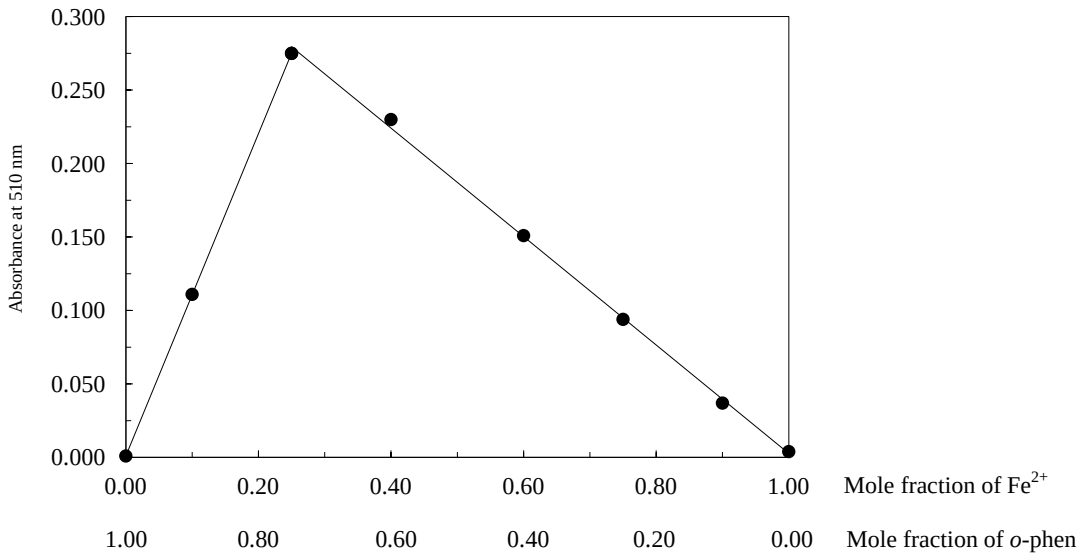


Figure 5.19 Job's plot studied on the complexation between Fe^{2+} and o-phenanthroline.

5.3.6 Analytical performances

Fe(II)-*o*-phenanthroline complex can be properly formed under pH 5.0 that was controlled by acetate bufffer. UV-Vis spectrometer at wavelength 510 nm was used for the detection. The complex solution was prepared by using different known volume of NH_2OH added with 500 µL of acetate buffer, pH 5.0 in volumetric flask 10 mL. Then 1 mL of 2×10^{-3} M Fe^{3+} and 2.5 mL of 1×10^{-3} M *o*-phenanthroline was added into the flask to produce Fe^{2+} and then form the Fe(II)-*o*-phenanthroline complex. Finally, the total volume was adjusted.

Calibration curve was plotted using the absorbance of Fe(II)-*o*-phenanthroline complexs that prepared from six concentrations of NH_2OH at 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L (Figure 5.20). The curve showed good linearity at r^2 =

0.9998. Intra-day and inter-day precision and recovery were measured at three concentration levels of NH_2OH, i.e., 0.05, 0.1, 2.0 mg/L. Precision of the method calculated using HORRAT equation was in the acceptable value of AOAC and recovery was in range75.3 to 105.4% (acceptable range = 75-120%). Table 5.5 and Table 5.6 show the performance of the method.

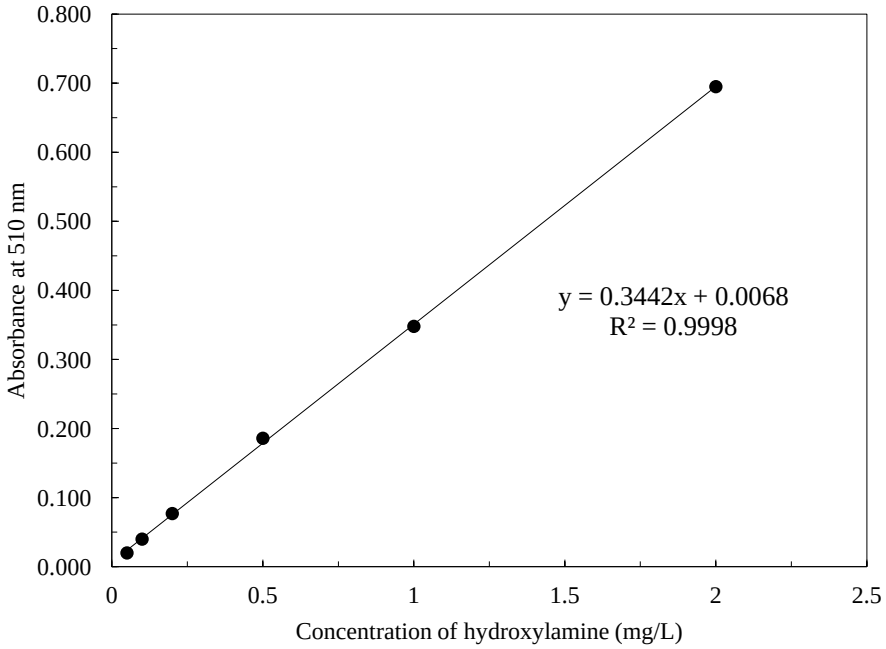


Figure 5.20 Calibration curve of standard NH_2OH for the colorimetric method.

Table 5.5 Analytical data of the colorimetric method for standard NH_2OH under the optimum conditions.

	Linear range (mg/L)	**Regression equation**	**Linearity (r^2)**	**DL (mg/L)**	**QL (mg/L)**
Fe(II)-*o*-phenanthroline	0.05-2.0	y = 0.3442x + 0.0068	0.9998	0.003	0.05

Table 5.6 Method precision and recovery for the colorimetric analysis of standard NH_2OH added into aqueous system (n = 10).

	0.05 mg/L		**0.1 mg/L**		**2.0 mg/L**	
	%RSD	**%Recovery**	**%RSD**	**%Recovery**	**%RSD**	**%Recovery**
Day 1	9.9	76.7	5.8	102.4	1.0	101.9
Day 2	10.3	67.6	4.1	106.7	1.4	106.5
Day 3	8.1	81.3	3.9	98.0	1.4	107.8
Inter-day	9.3	75.3	4.2	102.4	2.9	105.4

5.4 Hydroxylamine in rubber samples

Natural rubber samples in this project were obtained from Michelin and RTEC (project cooperation between Michelin and Rubber Technology Research Center, Mahidol university). Michelin samples were prepared by spraying NH_2OH solution into dried natural rubber. RTEC samples were prepared by mixing NH_2OH powder with dried natural rubber using rolling mill. The extraction steps were carried out by solid-liquid extraction which was developed by our lab and reflux which was proposed by Michelin lab. The amount of NH_2OH in rubber samples was determined by our three developed methods which were HPLC with cation-exchange column, HPLC with reversed-phase column, and colorimetry method.

5.4.1 Solid-liquid extraction (SLE)

Solid-liquid extraction method was performed by dissolving 1 g of natural rubber in dichloromethane and then extracting NH_2OH with suitable solvent, i.e., acetate buffer or DI water. The extractants were prepared for the analysis by using different methods shown in section 4.6.1.1, 4.6.2.1, and 4.6.3.1. The amount of NH_2OH in rubber samples was analyzed by our 3 developed methods and the results are shown in Table 5.7.

The first method is HPLC using cation-exchange column. The results showed that NH_2OH was not found in Michelin samples (the signal was lower than the detection limit.), but in RTEC samples, the amount of NH_2OH were significantly more than the added concentration. It is because of the side reaction between NH_2OH and mobile phase that affect the increasing of the signal with the increasing time. (The chromatograms are in Figure H.1 and I.1)

Second, the use of HPLC reversed-phase method showed similar results with the HPLC with cation exchange column that NH_2OH was not found in Michelin samples and the obtained signals were lower than the detection limit. For RTEC samples, the signal of acetone oxime was observed clearly at 6.5 minutes, but, the measured concentrations of NH_2OH were lower than the added concentration. (The chromatograms are in Figure H.2 and I.2)

The third method was the colorimetric method using the formation of Fe(II)-*o*-phenanthroline complex. A small amount of NH_2OH can be found in

Michelin samples at highest concentration added (3.0 g/kg) because of the high sensitivity of the method that detection limit was down to 0.003 mg/L. For RTEC rubber, NH_2OH can be detected in all samples, but the measured concentration was lower than the added concentration as well.

Table 5.7 Measured concentrations of $(NH_2OH)_2.SO_4$ in Michelin and RTEC samples extracted by SLE.

Added $(NH_2OH)_2.SO_4$ in 1 kg rubber (g)	**$(NH_2OH)_2.SO_4$ (g)**					
	1. HPLC cation-exchange column		**2. HPLC reversed-phase column**		**3. Colorimetry**	
	Michelin samples	RTEC samples	Michelin samples	RTEC samples	Michelin samples	RTEC samples
0.00	-	-	-	-	-	-
0.50	ND*	0.44 ± 0.13	ND	0.21 ± 0.01	ND	0.28 ± 0.03
0.80	ND	1.03 ± 0.15	ND	0.45 ± 0.02	ND	0.55 ± 0.05
2.00	ND	2.85 ± 0.12	ND	1.44 ± 0.04	ND	1.66 ± 0.05
3.00	ND	4.88	ND	2.20 ± 0.15	0.09 ± 0.01	2.44 ± 0.09

*N = Not detectable (the signal was lower than detection limit.)

5.4.2 Reflux

Michelin lab proposed the more drastic extraction techniques, such as, reflux, to extract NH_2OH from the matrix of rubber. The method was performed by boiling 3 g of rubber sample in 1 M sulfuric acid under reflux for 24 hr. The extractant was prepared for colorimetric analysis and then the amount of NH_2OH was calculated and shown in Table 5.8. NH_2OH can be extracted and found in both Michelin and RTEC samples. However, the low recovery of both samples were obtained.

Table 5.8 Measured concentrations of $(NH_2OH)_2.SO_4$ in Michelin and RTEC samples extracted by reflux.

Added $(NH_2OH)_2.SO_4$ in 1 kg rubber (g)	**$(NH_2OH)_2.SO_4$ (g)**	
	Michelin samples	RTEC samples
0.00	-	-
0.50	0.07	0.16
0.80	0.11	0.29
2.00	0.22	1.07
3.00	0.38	1.68

5.4.3 Summary

The percentage recoveries of the analysis of NH_2OH in Michelin samples and RTEC samples are summarized in Table 5.9 and Table 5.10, respectively. For Michelin sample, the low recoveries were obtained from both extraction methods that was lower than 2.9% by the SLE method and lower than 13.5% by the reflux method. For RTEC sample, the recoveries of the analysis with HPLC cation-exchange was obtained in the range of 88.3-162.6% that was more than 100%. It was because of the non-repeatable signal of the method resulting from the reaction between NH_2OH and acetonitrile (as described in section 5.1.2). For the analysis with HPLC reversed-phase and colorimetry, the recoveries were shown in the range of 41.2–82.8% by the SLE and 31.3-56.0% by the reflux. The low recovery presented in each sample may be because of (1) the inefficiency of the extraction method and (2) the transformation of NH_2OH in rubber. The solid-liquid extraction may not extract all of NH_2OH from the matrix of the rubber sample. The reflux is a drastic extraction technique under high concentration acid and heating system (high temperature) that can improve the extraction efficiency. However, the decomposition of NH_2OH may be occurred.

NH_2OH act as a viscosity stabilizer for inhibiting the storage hardening in natural rubber by reacting with the abnormal carbonyl group on the rubber chain. Therefore, NH_2OH may change to be another form, such as, oxime resulting in the decreasing of NH_2OH in the rubber samples. Moreover, the experiment to investigate the degradation process of NH_2OH in rubber samples was performed (Appendix K) and the result showed the loss of NH_2OH during storage. In the rubber sample,

Michelin sample that was obtained for the study was kept more than 1 year. It might be a reason that the results of NH_2OH were shown not detectable by the method used. Amount of NH_2OH content may continuously react with the component in the rubber. On the other hand, RTEC sample was freshly prepared samples by adding NH_2OH powder in the rubber and the results were still shown the content of NH_2OH from the method used.

Table 5.9 The summary of NH_2OH analysis analyzed from Michelin samples.

Added $(NH_2OH)_2.SO_4$ in 1 kg rubber (g)	**$(NH_2OH)_2.SO_4$, g/kg (%recovery)**			
	SLE			**Reflux** (Colorimetry)
	1. HPLC with cation-exchange column	2. HPLC with reversed-phase column	3. Colorimetry	
0.00	-	-	-	-
0.50	ND*	ND	ND	0.07 (*13.3%*)
0.80	ND	ND	ND	0.11 (*13.5%*)
2.00	ND	ND	ND	0.22 (*11.2%*)
3.00	ND	ND	0.09 (*2.9%*)	0.38 (*12.6%*)

*N = Not detectable (the signal was lower than detection limit.)

Table 5.10 The summary of NH_2OH analysis analyzed from RTEC samples.

Added $(NH_2OH)_2.SO_4$ in 1 kg rubber (g)	**$(NH_2OH)_2.SO_4$, g/kg (%recovery)**			
	SLE			**Reflux** (Colorimetry)
	1. HPLC with cation-exchange column	2. HPLC with reversed-phase column	3. Colorimetry	
0.00	-	-	-	-
0.50	0.44 (*88.3%*)	0.21 (*41.2%*)	0.28 (*55.8%*)	0.16 (*31.3%*)
0.80	1.03 (*128.7%*)	0.45 (*56.7%*)	0.55 (*68.5%*)	0.29 (*36.6%*)
2.00	2.85 (*142.7%*)	1.44 (*72.2%*)	1.66 (*82.8%*)	1.07 (*53.7%*)
3.00	4.88 (*162.6%*)	2.20 (*73.3%*)	2.44 (*81.2%*)	1.68 (*56.0%*)

CHAPTER VI
CONCLUSION

The amount of hydroxylamine (NH_2OH) in natural rubber were determined because of the effect to viscosity of the rubber. Natural rubber samples used in this study consisted of Michelin samples (natural rubber added with NH_2OH in form of solution) and Rubber Technology Research Centre (RTEC) samples (natural rubber added with NH_2OH in form of powder). Both samples were prepared at the same concentration by adding 0, 0.5, 0.8, 2, and 3 g of hydroxylamine sulfate into 1 kg of natural rubber. NH_2OH was extracted using two extraction methods, such as, solid-liquid extraction (SLE) and reflux. SLE method was carried out by swelling 1.0 g of rubber sample in dichloromethane and then extracting NH_2OH from organic/rubber phase using acetate buffer or deionized (DI) water. Reflux method was carried out by boiling 3.0 g of rubber sample under reflux in 1 M sulfuric acid. The extracts were prepared with different approaches to determine NH_2OH using the suitable analytical method. In this work, three analytical methods were developed, i.e., high performance liquid chromatography (HPLC) using cation-exchange column, HPLC using reversed-phase column, and colorimetry using Fe(II)-*o*-phenanthroline complex formation.

First, HPLC with cation-exchange column, the method was carried out on Synchrom S300 column (250 mm × 4.6 mm) by using (60:40 v/v) acetate buffer pH 5.0 (10 mM acetic acid):acetonitrile as a mobile phase. However, after the processes of optimization and method validation, the results showed poor repeatability of peak area that was significantly increased with increasing time and the recoveries were unacceptable. It is due to the undesired oxime product formation between NH_2OH and acetonitrile. Therefore, this method was not suitable for determining NH_2OH and it needs to be solved and developed.

Second, the analysis of NH_2OH using HPLC reversed-phase column was performed depended on the reaction between NH_2OH and acetone to form acetone oxime product. The separation was carried out on LiChroCART® (250 mm × 4 mm,

RP-18 (5 µm)) column using 10:90 (%v/v) methanol: DI water as a mobile phase. UV-Vis detector at 230 nm was used. The method performance showed linearity in the range of 0.5 to 50.0 mg/L with r^2 = 0.9996. Detecion limit (DL) of the method was 0.16 mg/L.

Finally, the colorimetric method was performed by the indirect analysis using Fe(II)-*o*-phenanthroline complex formation. The suitable controlling pH was at 5.0. The complex was measured by UV-Vis spectrophotometer at 510 nm. Linearity of the method was in the range of 0.05 to 2 mg/L with R^2 = 0.9998. DL was 0.003 mg/L.

Therefore, NH_2OH in rubber samples can be analyzed by two suitable methods that were HPLC reversed-phase column and colorimetry. For Michelin samples, the recovery showed lower than 2.9% by SLE and lower than 13.5% by reflux. The higher recovery in reflux was obtained because reflux is a stronger extraction method using high concentration of sulfuric acid. For RTEC samples, the recovery showed in the range of 41.2-82.8% by SLE and 31.3-56.0% by reflux. The low recovery was resulted from the degradation of NH_2OH in rubber.

Suggestions for future work

1. Colorimetric method using Fe(II)-*o*-phenanthroline complex formation is more preferable for applying in the industrial scale because it is a simple and low cost technique. However, the interference from other antioxidants in the rubber have to be concerned.

2. The efficiency of the extraction method can be improved by modifying the method with centrifugation in order to reduce the emulsion of rubber-water phase.

3. Thermogravimetric analysis (TGA) is the interesting technique to determine the degradation of NH_2OH in rubber by measuring the weight loss of the rubber after decomposition of NH_2OH with high temperature.

4. For the work with Michelin company, the relationship between concentration of added NH_2OH in the rubber and the amount of NH_2OH presented in the extract was linearly correlated. NH_2OH was partially extracted from the rubber sample and the correlation would be provide a good information to find the content of added NH_2OH in the rubber sample.

REFERENCES

Abe, A. (1998). Liquid color developer for silver halide color photosensitive materials and processing method using the same: Google Patents.

Adhikamsetty, R. K., Gollapalli, N. R., & Jonnalagadda, S. B. (2008). Complexation kinetics of Fe2+ with 1,10-phenanthroline forming ferroin in acidic solutions. International Journal of Chemical Kinetics, 40(8), 515-523. doi: 10.1002/kin.20336

Afkhami, A., Madrakian, T., & Maleki, A. (2005). Spectrophotometric determination of hydroxylamine and nitrite in mixture in water and biological samples after micelle-mediated extraction. Analytical Biochemistry, 347(1), 162-164. doi: 10.1016/j.ab.2005.09.018

Afkhami, A., Madrakian, T., & Maleki, A. (2006). Indirect kinetic spectrophotometric determination of hydroxylamine based on its reaction with iodate. Analytical Sciences, 22(2), 329-331. doi: 10.2116/analsci.22.329

AOAC. (2013). Guidelines for Dietary Supplements and Botanicals. 1-32. http://www.eoma.aoac.org/app_k.pdf

Avagyan, R., Sadiktsis, I., Thorsén, G., Östman, C., & Westerholm, R. (2013). Determination of benzothiazole and benzotriazole derivates in tire and clothing textile samples by high performance liquid chromatography-electrospray ionization tandem mass spectrometry. Journal of Chromatography A, 1307, 119-125. doi: 10.1016/j.chroma.2013.07.087

BASF. (2012). Hydroxylamine (≤50% in aqueous solution). GPS Safety Summary. from http://www.safety-summaries.basf.com/group/corporate/safety-summaries/en/literature-document:/GPS+Safety+Summaries--Hydroxylamine+50+in+aqueous+solution-English.pdf

Benvidi, A., Jahanbani, S., Akbari, A., & Zare, H. R. (2015). Simultaneous determination of hydrazine and hydroxylamine on a magnetic bar carbon paste electrode modified with reduced graphene oxide/Fe3O4 nanoparticles and a heterogeneous mediator. Journal of Electroanalytical Chemistry, 758, 68-77. doi: 10.1016/j.jelechem.2015.10.008

Benvidi, A., Kakoolaki, P., Gorji, A. R., Mazloum-Ardakani, M., Zare, H. R., & Vafazadeh, R. (2013). Application of Co(ii) complex multi-wall carbon nanotube modified carbon paste electrodes for electrocatalytic determination of hydroxylamine. Analytical Methods, 5(23), 6649-6655. doi: 10.1039/c3ay40993g

Bergendorff, O., Persson, C., & Hansson, C. (2006). High-performance liquid chromatography analysis of rubber allergens in protective gloves used in health care. Contact Dermatitis, 55(4), 210-215. doi: 10.1111/j.1600-0536.2006.00912.x

Bergendorff, O., Persson, C. M. L., & Hansson, C. (2004). HPLC analysis of alkyl thioureas in an orthopaedic brace and patch testing with pure ethylbutylthiourea. Contact Dermatitis, 51(5-6), 273-277. doi: 10.1111/j.0105-1873.2004.00468.x

Blanco, M., Coello, J., Iturriaga, H., Maspoch, S., & Bertran, E. (1997). Determination of accelerators and antioxidants in vulcanized rubber by fourier transform infrared spectrophotometry. Analytica Chimica Acta, 353(2-3), 351-358. doi: 10.1016/S0003-2670(97)87797-5

Bourke, G. C. M., Stedman, G., & Wade, A. P. (1983). The spectrophotometric determination of hydroxylamine alone and in the presence of hydrazine by flow injection analysis. Analytica Chimica Acta, 153(C), 277-280. doi: 10.1016/S0003-2670(00)85514-2

Bowman, J., Tang, L., & Silverman, C. E. (2000). Analysis of small amines in pharmaceuticals by capillary ion electrophoresis with conductivity detection. Journal of Pharmaceutical and Biomedical Analysis, 23(4), 663-669. doi: 10.1016/S0731-7085(00)00341-1

Brown, G.A., Cansfield, J.E., Congreve, M.S., PICKWORTH, M., & TEHAN, B.G. (2014). Bicyclic aza compounds as muscarinic m1 receptor agonists: Google Patents.

Deepa, B., Balasubramanian, N., & Nagaraja, K. S. (2004). Spectrophotometric determination of hydroxylamine and its derivatives in pharmaceuticals. Chemical and Pharmaceutical Bulletin, 52(12), 1473-1475. doi: 10.1248/cpb.52.1473

Dias, F., Olojola, A. S., & Jaselskis, B. (1979). Spectrophotometric determination of micro amounts of hydrazine and hydroxylamine alone and in the presence of each other. Talanta, 26(1), 47-49. doi: 10.1016/0039-9140(79)80152-6

Fernando, P. N., Egwu, I. N., & Hussain, M. S. (2002). Ion chromatographic determination of trace hydroxylamine in waste streams generated by a pharmaceutical reaction process. Journal of Chromatography A, 956(1-2), 261-270. doi: 10.1016/S0021-9673(02)00145-0

Foroughi, M. M., Beitollahi, H., Tajik, S., Hamzavi, M., & Parvan, H. (2014). Hydroxylamine electrochemical sensor based on a modified carbon nanotube paste electrode: Application to determination of hydroxylamine in water samples. International Journal of Electrochemical Science, 9(6), 2955-2965.

George, M., Balasubramanian, N., & Nagaraja, K. S. (2007). Spectrophotometric determination of hydroxylamine and its derivatives in drug formulation using methyl red. Indian Journal of Chemical Technology, 14(4), 412-416.

Guerra, R. M., Marín, M. L., Sánchez, A., & Jiménez, A. (2002). Analysis of citrates and benzoates used in poly(vinyl chloride) by supercritical fluid extraction and gas chromatography. Journal of Chromatography A, 950(1-2), 31-39. doi: 10.1016/S0021-9673(02)00051-1

Guzowski Jr, J. P., Golanoski, C., & Montgomery, E. R. (2003). A gas chromatographic method for the indirect determination of hydroxylamine in pharmaceutical preparations: Conversion into nitrous oxide. Journal of Pharmaceutical and Biomedical Analysis, 33(5), 963-974. doi: 10.1016/S0731-7085(03)00433-3

Ikarashi, Y., & Kaniwa, M. A. (2000). Determination of p-phenylenediamine and related antioxidants in rubber boots by high performance liquid chromatography. Development of an analytical method for N-(1-methylheptyl)-N′-phenyl-p-phenylenediamine. Journal of Health Science, 46(6), 467-473.

Khatoon, S. S. (2014). Oximes. from https://chemistry.com.pk/organic-chemistry/oximes-short-introduction/

Korte, W. D. (1992). Determination of hydroxylamine in aqueous solutions of pyridinium aldoximes by high-performance liquid chromatography with UV and fluorometric detection. Journal of Chromatography A, 603(1-2), 145-150. doi: 10.1016/0021-9673(92)85355-W

Li, Z., Chen, R., Jin, W., & Xing, W. (2010). Catalytic mechanism and reaction pathway of acetone ammoximation to acetone oxime over TS-1. Korean Journal of Chemical Engineering, 27(5), 1423-1427. doi: 10.1007/s11814-010-0236-1

Marasco, Martin. (1926). Hydroxylamine Hydrochloride for the Quick Estimation of Acetone. Industrial & Engineering Chemistry, 18(7), 701-702. doi: 10.1021/ie50199a010

Moldovan, Z., & Alexandrescu, L. (2002). Derivative spectrophotometry in the determination of phenyl-beta-naphthylamine used as an antioxidant in rubber mixtures. Anal Bioanal Chem, 374(1), 46-50. doi: 10.1007/s00216-002-1297-8

Moldovan, Z., Stoica, C., Hillebrand, M., Alexandrescu, L., & MacOvescu, G. (2005). Spectrofluorimetric determination of phenyl-β-naphthylamine used as rubber antioxidant. Analytical and Bioanalytical Chemistry, 381(7), 1381-1386. doi: 10.1007/s00216-004-2938-x

Ngolemasango, F., Ehabe, E., Aymard, C., Sainte-Beuve, J., Nkouonkam, B., & Bonfils, F. (2003). Role of short polyisoprene chains in storage hardening of natural rubber. Polymer International, 52(8), 1365-1369. doi: 10.1002/pi.1225

Park, H. B., Pyo, S. H., Hong, S. S., & Kim, J. H. (2001). Optimization of the hydroxylamine cleavage of an expressed fusion protein to produce a recombinant antimicrobial peptide. Biotechnology Letters, 23(8), 637-641. doi: 10.1023/A:1010358131124

Peng, S. X., Strojnowski, M. J., Hu, J. K., Smith, B. J., Eichhold, T. H., Wehmeyer, K. R., . . . Almstead, N. G. (1999). Gas chromatographic-mass spectrometric analysis of hydroxylamine for monitoring the metabolic hydrolysis of metalloprotease inhibitors in rat and human liver microsomes. Journal of Chromatography B: Biomedical Sciences and Applications, 724(1), 181-187. doi: 10.1016/S0378-4347(98)00557-X

Pohl, Christopher A., Stillian, John R., & Jackson, Peter E. (1997). Factors controlling ion-exchange selectivity in suppressed ion chromatography. Journal of Chromatography A, 789(1–2), 29-41. doi: http://dx.doi.org/10.1016/S0021-9673(97)00705-X

Prokai, A. M., & Ravichandran, R. K. (1994). Simultaneous analysis of hydroxylamine, N-methylhydroxylamine and N,N-dimethylhydroxylamine by ion chromatography. Journal of Chromatography A, 667(1-2), 298-303. doi: 10.1016/0021-9673(94)89079-X

Proll, P. J., & Sutcliffe, L. H. (1961). Dissociation constants of some inorganic acetates in anhydrous acetic acid. Transactions of the Faraday Society, 57(0), 1078-1087. doi: 10.1039/TF9615701078

PubChem. (2016). Hydroxylamine. Retrieved 17 March, 2016, from https://pubchem.ncbi.nlm.nih.gov/compound/hydroxylamine#section=Top

Scherr, O.L. (1948). Hydroxylamine stabilized fatty materials: Google Patents.

Seike, Y., Fukumori, R., Senga, Y., Oka, H., Fujinaga, K., & Okumura, M. (2004). A Simple and Sensitive Method for the Determination of Hydroxylamine in Fresh-Water Samples Using Hypochlorite Followed by Gas Chromatography. Analytical Sciences, 20(1), 139-142. doi: 10.2116/analsci.20.139

Shabani-Nooshabadi, M., & Tahernejad-Javazmi, F. (2015). Electrocatalytic Determination of Hydroxylamine in the Presence of Thiosulfate in Water and Wastewater Samples Using a Nanostructure Modified Carbon Paste Electrode. Electroanalysis, 27(7), 1733-1741. doi: 10.1002/elan.201500046

Shishehbore, M. R., Zare, H. R., Nematollahi, D., & Saber-Tehrani, M. (2011). Electrocatalytic oxidation and differential pulse voltammetric determination of hydroxylamine using a 4-hydroxy-2-(triphenylphosphonio) phenolate carbon nanotubes modified electrode. Analytical Methods, 3(2), 306-313. doi: 10.1039/c0ay00451k

Thaimac. (2015). Standard Thai Rubber (STR). Retrieved 17 March, 2016, from http://www.thaimac.co.th/product/standard-thai-rubber-str/

Yongzhou, W., Pingyue, W., Beilong, Z., Honghai, H., Li, D., Fuquan, Z., . . . Rizhong, Z. (2012) Effect of viscosity stabilizer on the properties and structure of raw constant viscosity rubber. Vol. 557-559. Advanced Materials Research (pp. 947-951).

Zhang, B., Deng, W., Huang, H., Ding, L., Wang, Y., & Wang, P. Y. (2012) Study on effective storage time of constant viscosity natural rubber. Vol. 396-398. Advanced Materials Research (pp. 1879-1884).

ทัศนกุล, ปรีดิ์เปรม. (2557). ปัจจัยที่ใช้ในการควบคุมคุณภาพยางแท่งจากยางแห้ง. PARA RUBBER ELECTRONIC BULLETIN, 35(2), 20.

พัฒนกุล, วิภาวี. (2554). ยางธรรมชาติและยางสังเคราะห์. Retrieved 17 มีนาคม, 2559, from http://www.rubberthai.com/book/file/98.pdf

มหาวิทยาลัยสงขลานครินทร์. (2553). ทฤษฎีและหลักการที่เกี่ยวข้อง. Retrieved 17 มีนาคม, 2559, from http://kb.psu.ac.th/psukb/bitstream/2553/2293/7/272796_ch2.pdf

APPENDICES

APPENDIX A
THE PRODUCTION PROCESS OF CONSTANT VISCOSITY RUBBER

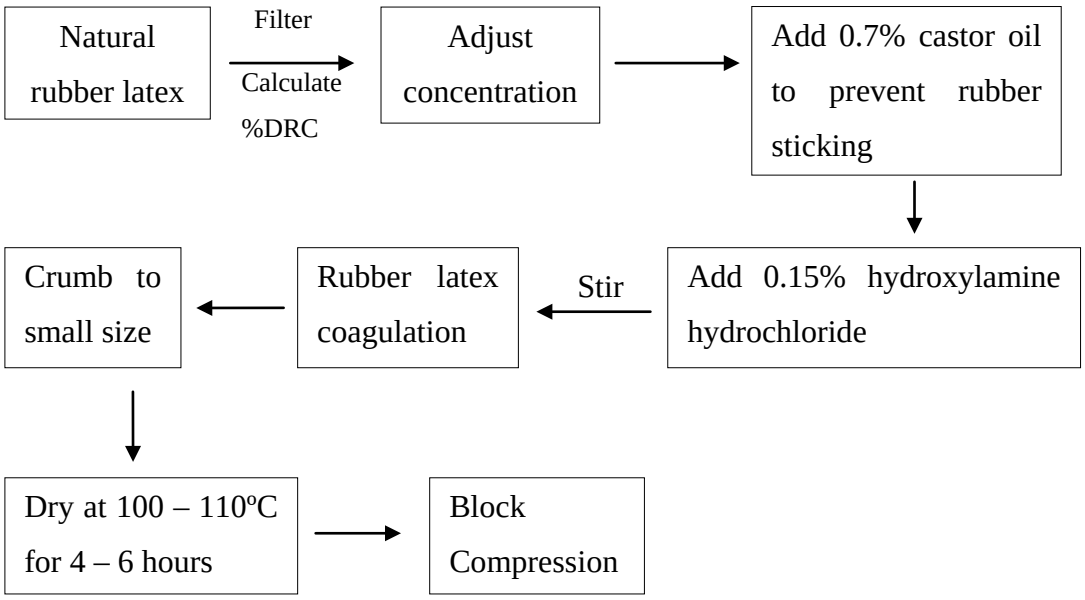


Figure A.1 Production procedures of CV rubber by using natural rubber latex.

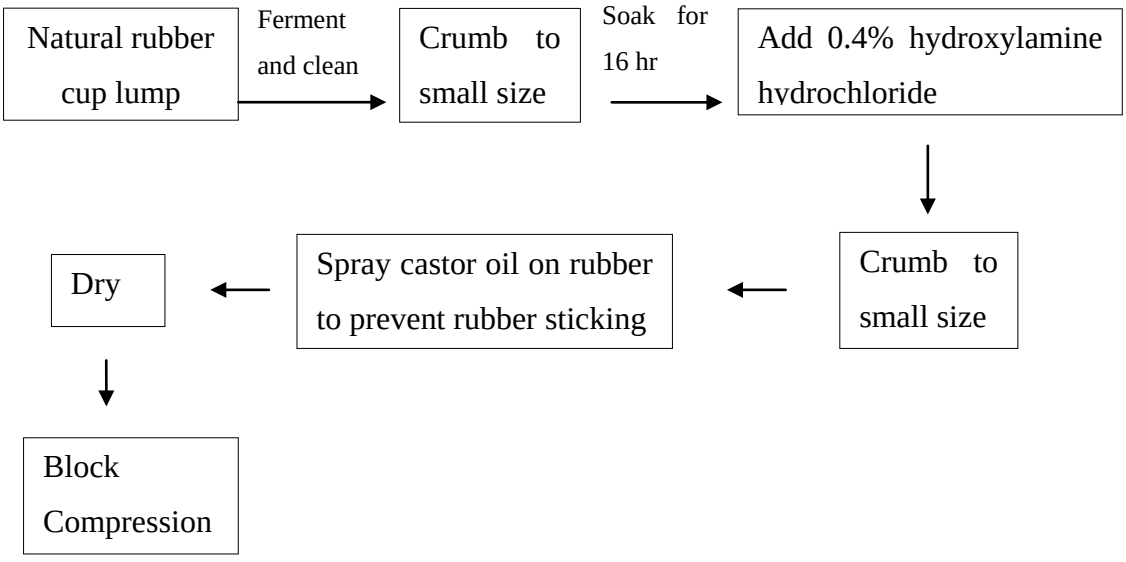


Figure A.2 Production procedures of CV rubber by using natural rubber cup lump.

APPENDIX B

MOBILE PHASE SELECTION IN HPLC CATION-EXCHANGE

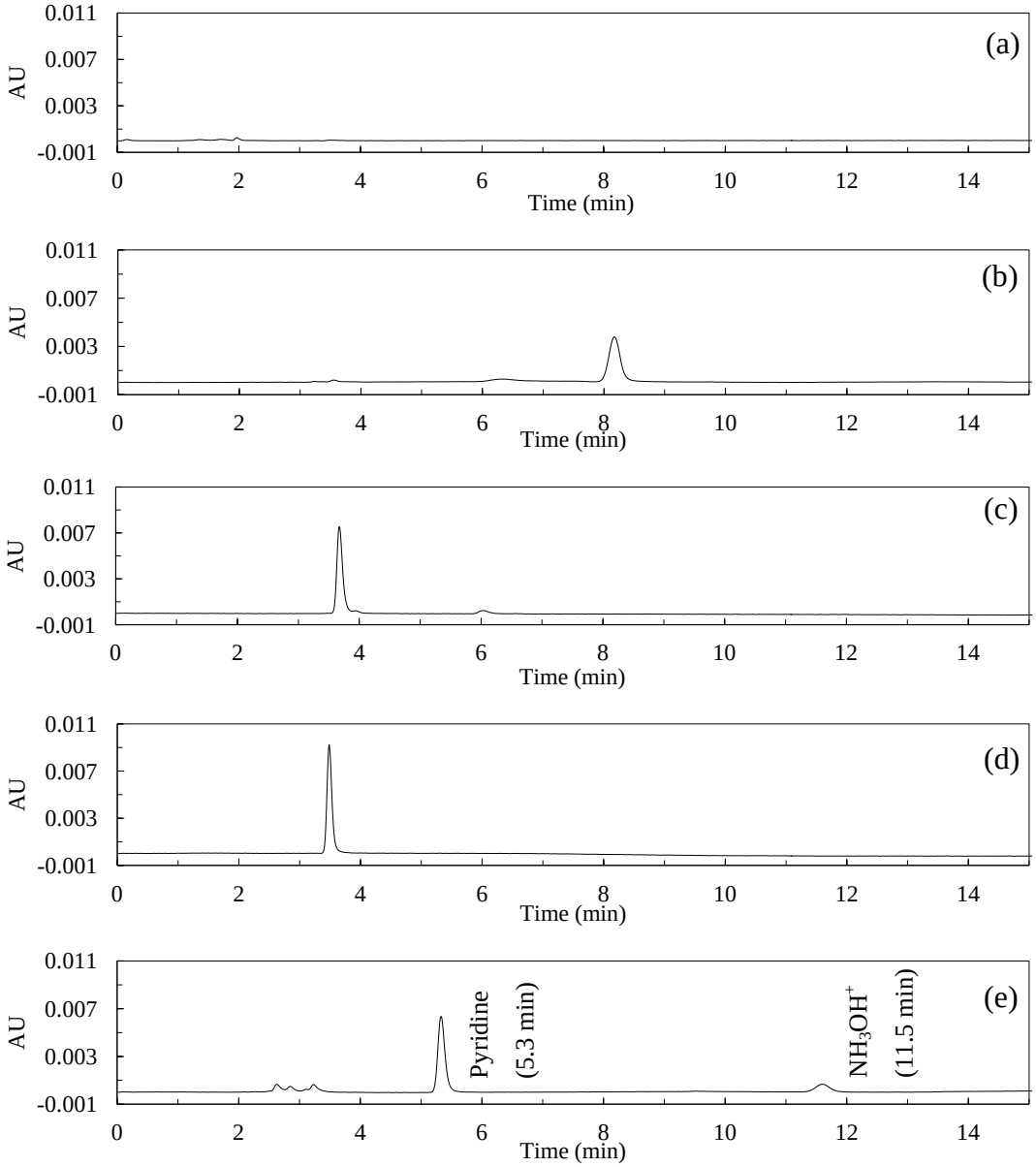


Figure B.1 HPLC chromatograms of 10 mg/L of pyridine and 50 mg/L of NH_2OH using different mobile phase: (a) 10 mM nitric acid; (b) 10 mM acetic acid; (c) 10 mM sulfuric acid; (d) 10 mM phosphoric acid; (e) 60:40 (%v/v) acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile.

APPENDIX C
ABSORPTION SPECTRUM

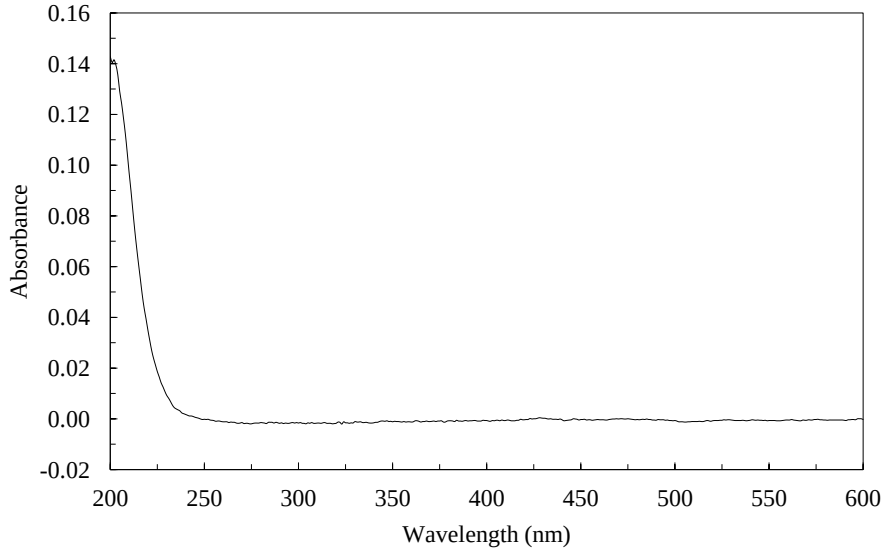


Figure C.1 Absorption spectrum (in range 200-600 nm) of 50 mg/L of NH_2OH in 60:40 (%v/v) acetate buffer pH 4.5 (10 mM acetic acid):acetonitrile after set zero with solvent blank.

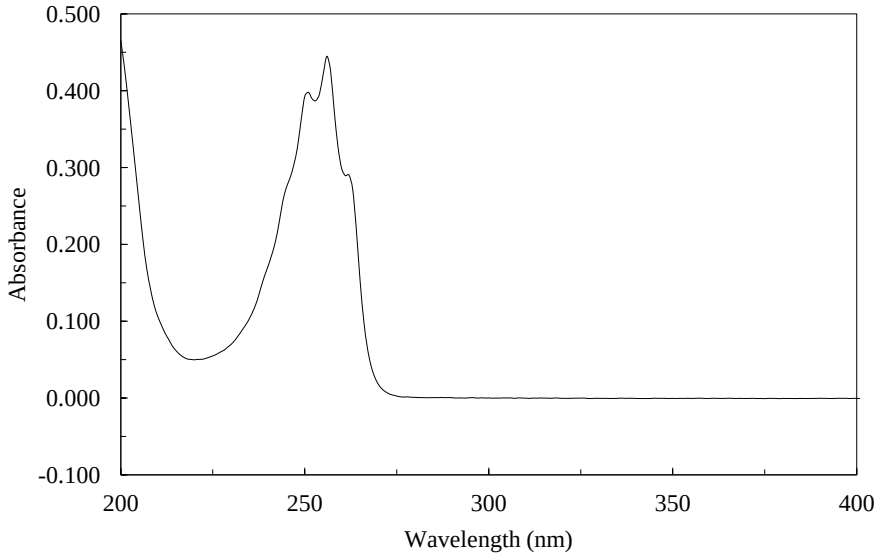


Figure C.2 Absorption spectrum (in range 200-400 nm) of pyridine in DI water.

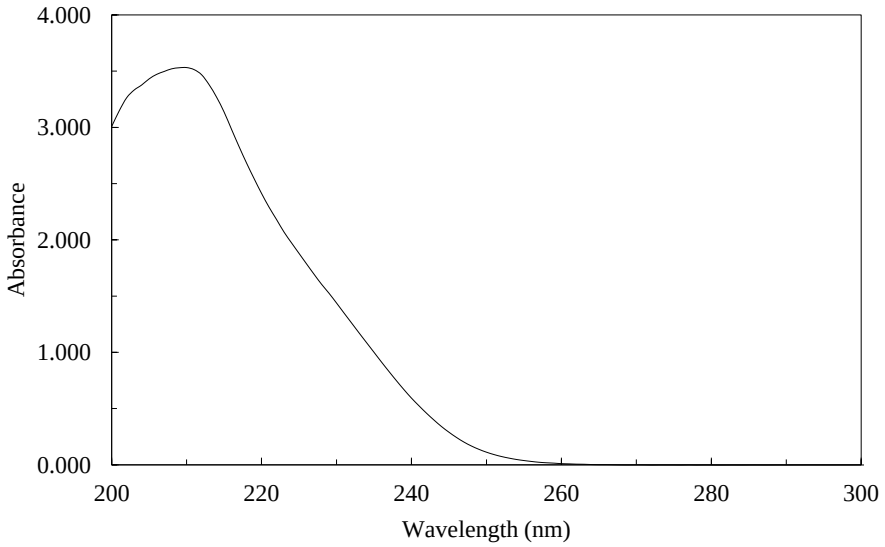


Figure C.3 Absorption spectrum scanning in the range of 200-300 nm of acetone oxime prepared from NH_2OH 100 mg/L.

APPENDIX D
THE STUDY OF ACETONE OXIME REACTION

Effect of reaction temperature

The reactions of acetone oxime (500 mg/L of NH_2OH) were controlled at different temperatures; 25, 30, 40, 50, 60, 70, and 80ºC using water bath. The products were analyzed with HPLC reversed-phase method. The results are shown in Figure D.1.

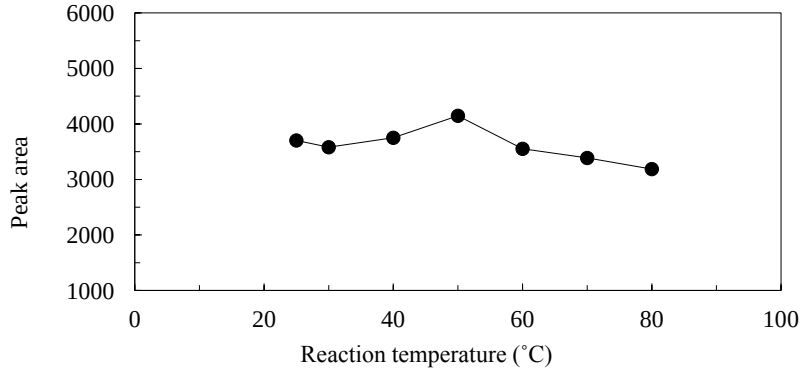


Figure D.1 Effect of reaction temperature to the formation of acetone oxime.

Effect of reaction time

The acetone oxime products (500 mg/L of NH_2OH) were collected from the reaction at different time; 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 minutes. The products were analyzed with HPLC reversed-phase and the results show in Figure D.2.

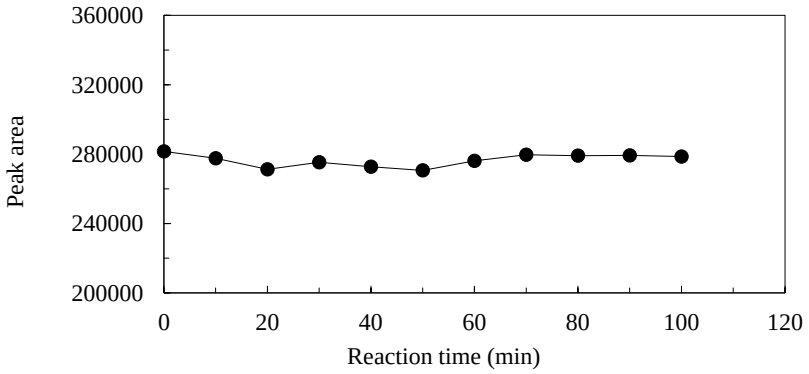


Figure D.2 Effect of reaction time to the formation of acetone oxime.

APPENDIX E

OPTIMIZATION OF Fe(II)-*o*-PHENANTHROLINE COMPLEX FORMATION

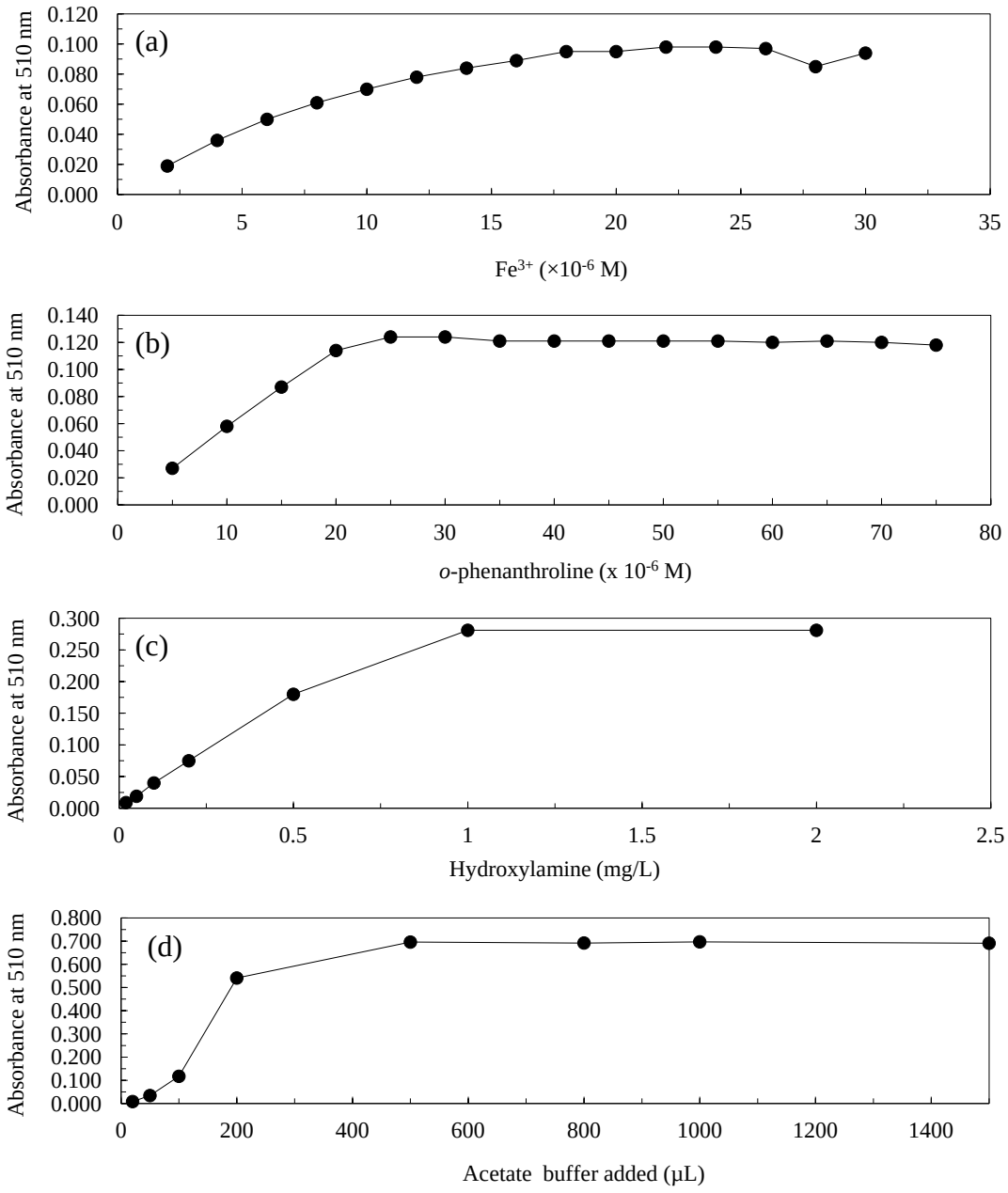
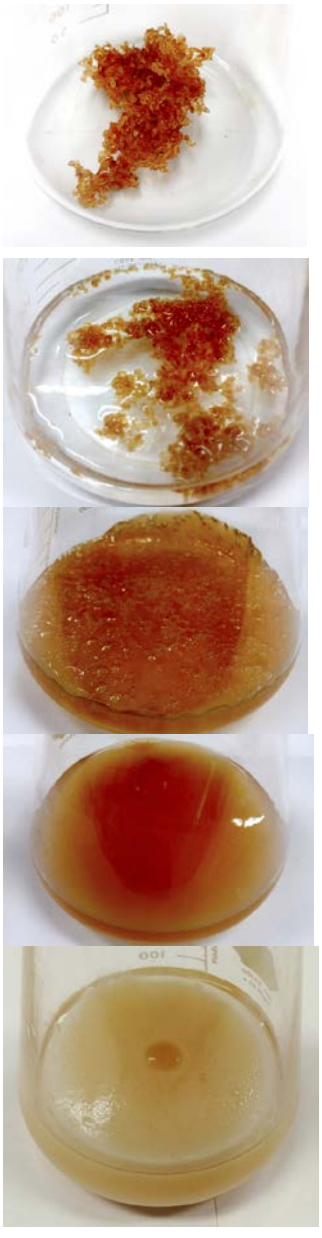


Figure E.1 Optimization of colorimetric method by varing of different reagents (a) Fe^{3+}, (b) o-phenanthroline, (c) hydroxylamine, and (d) acetate buffer.

APPENDIX F
SOLID LIQUID EXTRACTION STEP OF EXTRACTION OF NH_2OH IN NR SAMPLES



Rubber extraction processes

1) Natural rubber sample was cut into small piece, 3 mm × 3 mm
2) 1 g of sample was weighed and placed in 250 mL Erlenmeyer flask
3) 50 mL of dichloromethane was added into the flask.
4) The sample was sonicated for 1 hr.
5) Then the sample was shaken with reciprocating shaker for 5 hr. (shaking rate = 100 rounds/min)
6) 20 mL of DI water was added into the flask and then shaked for 1 hr (shaking rate = 125 rounds/min) to extract NH_2OH from organic phase.
7) The extracted solution was prepared for NH_2OH analysis

Figure F.1 Natural rubber sample during solid-liquid extraction

APPENDIX G

REFLUX APPARATUS FOR RUBBER EXTRACTION

Figure G.1 Picture of reflux set-up for extraction of natural rubber

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APPENDIX H
MICHELIN SAMPLES

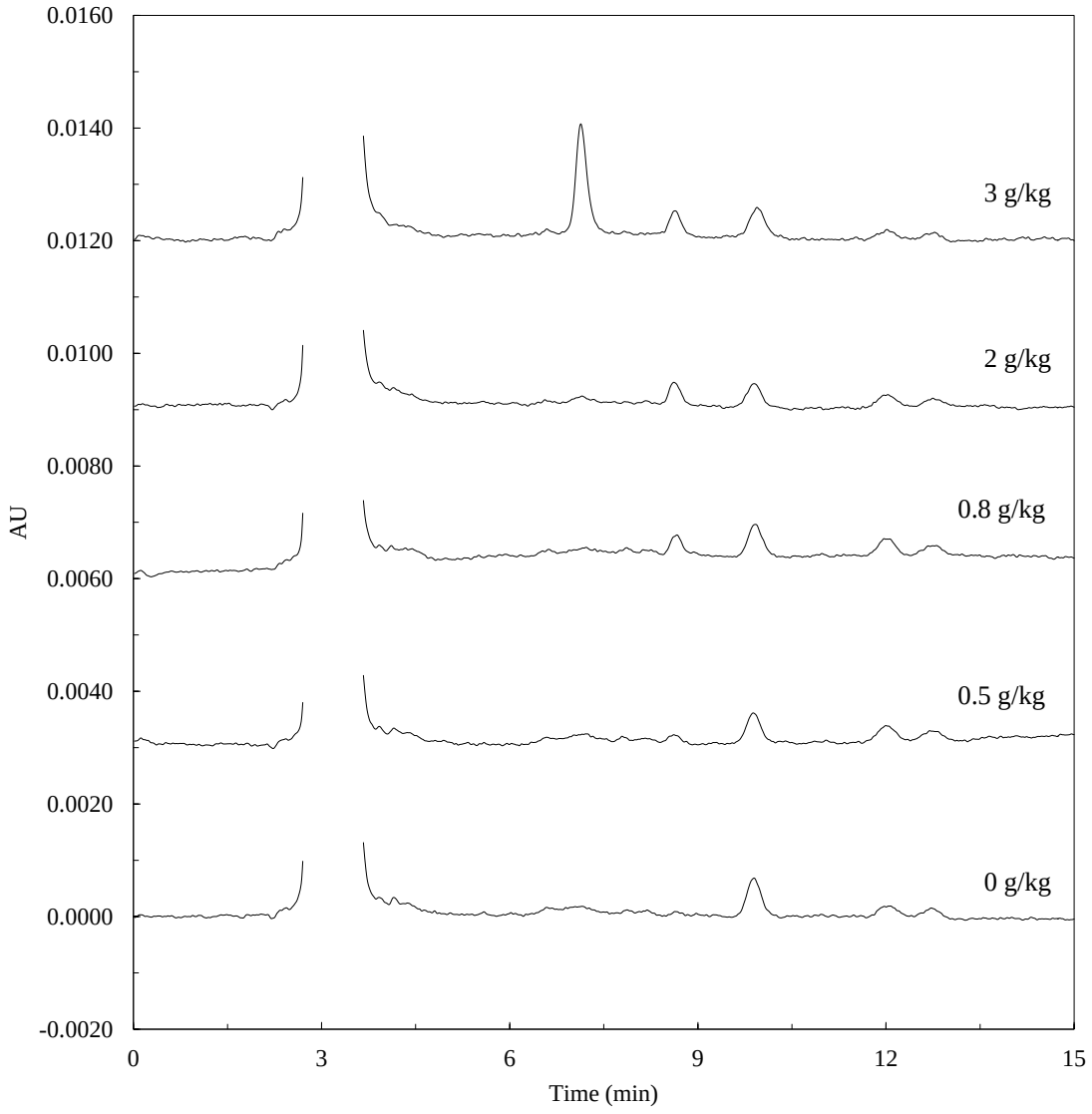

Figure H.1 Chromatograms of Michelin samples analysis obtained from HPLC with cation-exchange column.

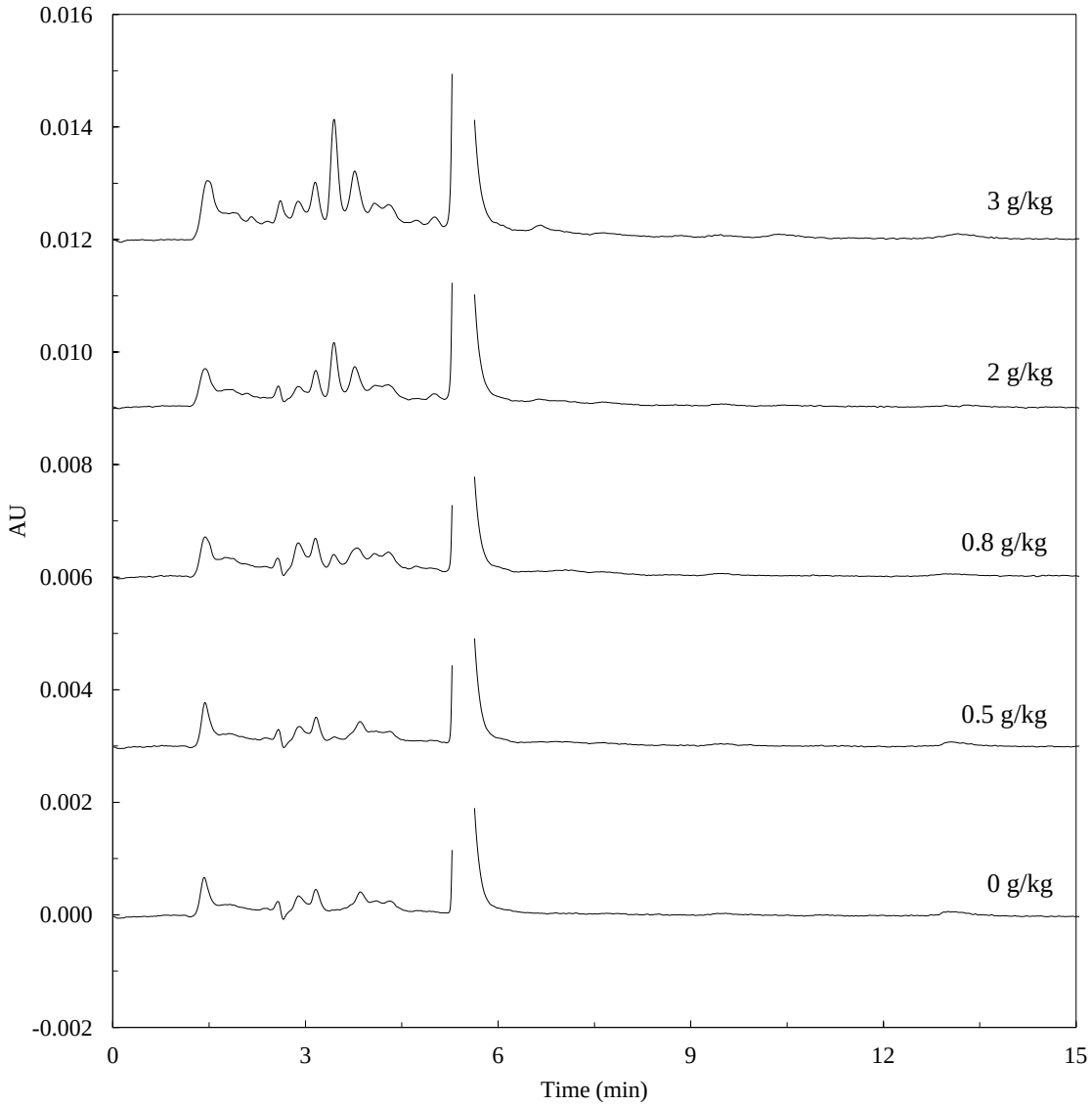

Figure H.2 Chromatograms of Michelin samples analysis obtained from HPLC with reversed-phase column.

APPENDIX I
RTEC SAMPLES

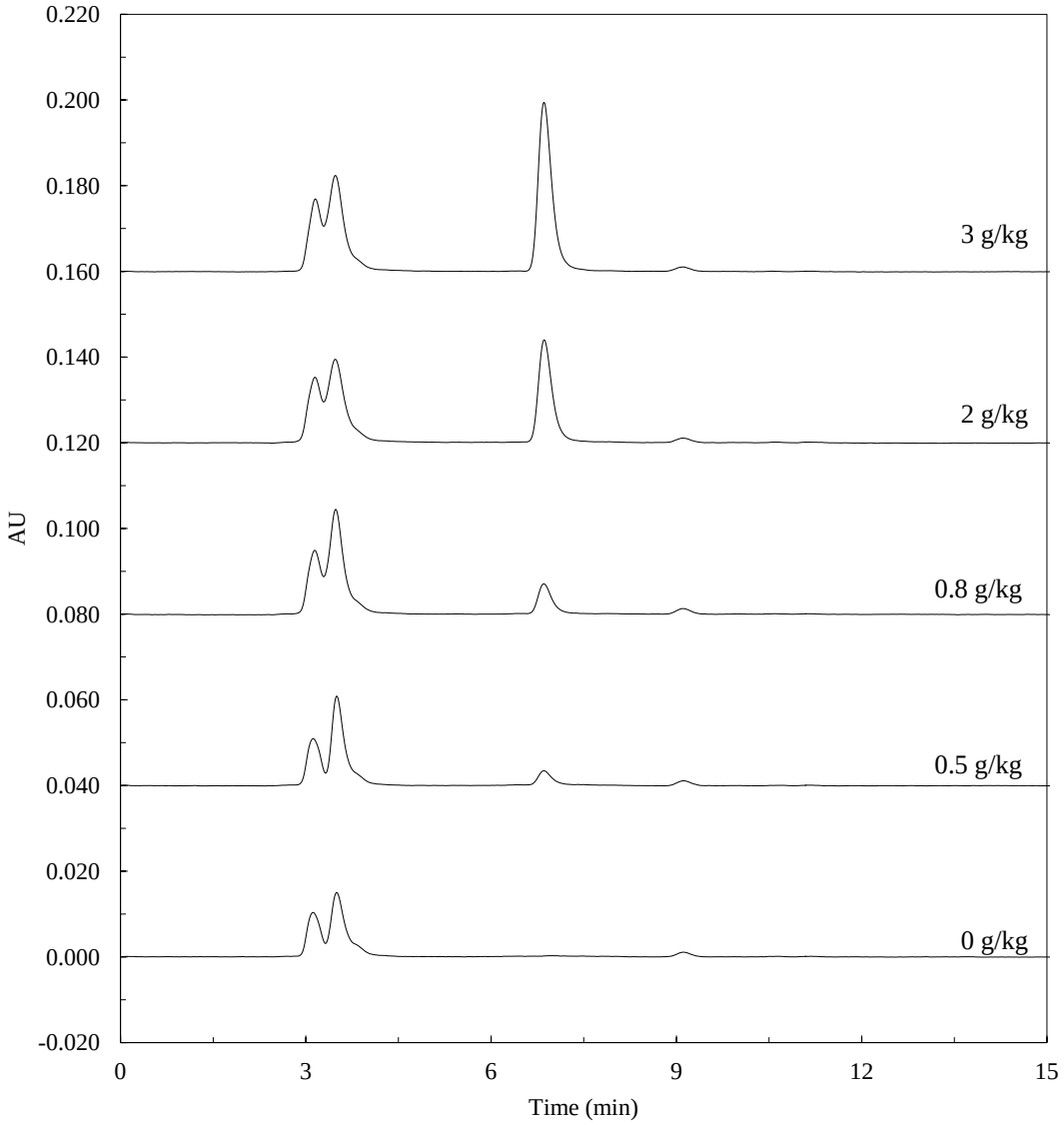

Figure I.1 Chromatograms of RTEC samples analysis obtained from HPLC with cation-exchange column.

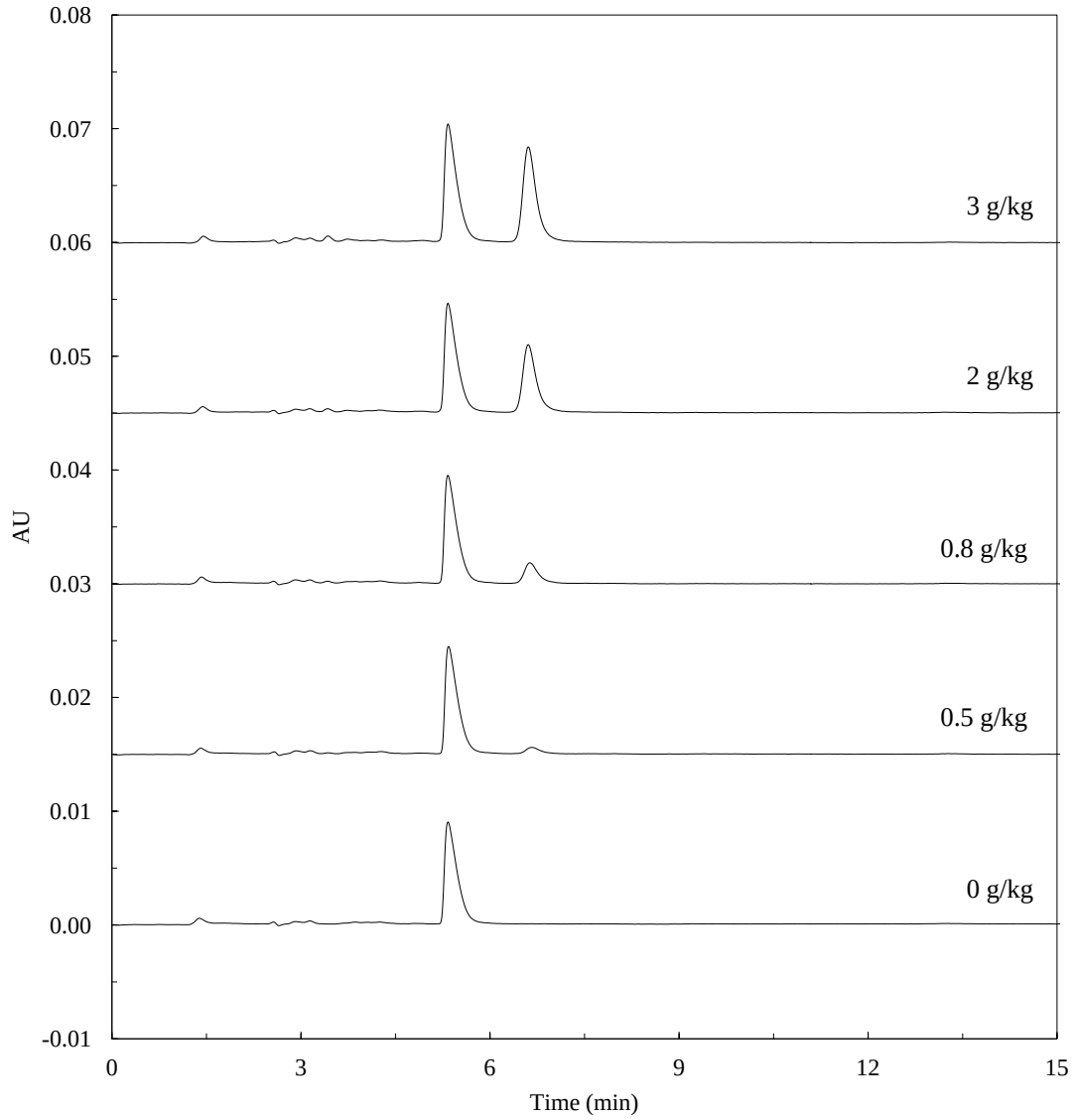

Figure I.2 Chromatograms of RTEC samples analysis obtained from HPLC with reversed-phase column.

APPENDIX J
DATA OF NH_2OH ANALYSIS IN RUBBER SAMPLES

Table A.1 Data of NH_2OH analysis in rubber samples using HPLC with cation-exchange method.

Added $(NH_2OH)_2.SO_4$ in 1 kg rubber (g)	**$(NH_2OH)_2.SO_4$ (g)**			**$\bar{X}$ (g/kg)**	**RSD (%)**
	NO.1	**NO.2**	**NO.3**		
0.5	0.31	0.57	0.45	0.44 ± 0.13	30.4
0.8	1.00	1.19	0.90	1.03 ± 0.15	14.5
2	2.74	2.85	2.97	2.85 ± 0.12	4.1
3	4.88	N	N	4.88	-

Table A.2 Data of NH_2OH analysis in rubber samples using HPLC with reversed-phase column.

Added $(NH_2OH)_2.SO_4$ in 1 kg rubber (g)	**Day**	**Found (g/kg)**			**$\bar{X}$ (g/kg)**	**RSD (%)**
		NO.1	**NO.2**	**NO.3**		
0.5	1	0.24	0.22	0.19	0.22 ± 0.02	9.8
	2	0.29	0.15	0.16	0.20 ± 0.08	39.1
	3	N	0.15	0.25	0.20 ± 0.07	35.9
				Average	0.21	
				SD	0.01	
				RSD	5.1	
0.8	1	0.29	0.71	0.45	0.48 ± 0.21	43.3
	2	0.43	0.44	0.47	0.44 ± 0.02	5.2
	3	0.34	0.41	0.56	0.44 ± 0.11	25.3
				Average	0.45	
				SD	0.02	
				RSD	5.5	
2.0	1	1.43	1.54	1.46	1.47 ± 0.05	3.7
	2	1.38	1.52	1.45	1.45 ± 0.07	4.8
	3	1.48	1.42	1.32	1.41 ± 0.08	5.5
				Average	1.44	
				SD	0.04	
				RSD	2.4	
3.0	1	2.40	2.30	2.41	2.37 ± 0.06	2.5
	2	2.13	2.12	2.04	2.10 ± 0.05	2.4
	3	2.21	2.14	2.04	2.13 ± 0.09	4.0
				Average	2.20	
				SD	0.15	
				RSD	6.9	

Table A.3 Data of NH_2OH analysis in rubber samples using colorimetric method (SLE).

Exact Amount (g/kg)	**Day**	**Found (g/kg)**			**$\bar{X}$ (g/kg)**	**RSD (%)**
		NO.1	**NO.2**	**NO.3**		
0.5	1 (Day 5)	0.27	0.23	0.24	0.24 ± 0.02	9.5
	2 (Day 27)	0.22	0.37	0.31	0.30 ± 0.08	24.9
	3 (Day 57)	0.29	0.29	0.28	0.29 ± 0.01	2.3
				Average	0.28	
				SD	0.03	
				RSD	11.0	
0.8	1	0.49	0.49	0.51	0.49 ± 0.01	2.6
	2	0.56	0.66	0.55	0.59 ± 0.06	10.1
	3	0.49	0.51	0.68	0.56 ± 0.10	18.5
				Average	0.55	
				SD	0.05	
				RSD	8.8	
2.0	1	1.65	1.82	1.68	1.71 ± 0.09	5.2
	2	1.57	1.56	1.71	1.61 ± 0.09	5.4
	3	1.62	1.66	1.64	1.64 ± 0.02	1.0
				Average	1.66	
				SD	0.05	
				RSD	3.2	
3.0	1	2.45	2.62	2.53	2.53 ± 0.09	3.4
	2	2.58	2.40	2.31	2.43 ± 0.14	5.7
	3	2.29	2.45	2.30	2.35 ± 0.09	3.8
				Average	2.44	
				SD	0.09	
				RSD	3.8	

APPENDIX K

THE DEGRADATION PROCESS OF NH_2OH IN RTEC RUBBER

The experimentation for investigating the degradation of NH_2OH in natural rubber sample was performed by measuring NH_2OH in RTEC sample (the freshly prepared sample) at different storaged time, i.e., 5, 27, 57, 80, 132, and 291 days. Colorimetric method was applied for detecting NH_2OH in this study. The graph between the amount of NH_2OH in rubber sample and storaged time (day) was plotted as shown in Figure K.1. The result shows the decreasing of NH_2OH with time from 2.53 g/kg (on day 5) to 1.23 g/kg (on day 291) that can indicate the degradation of NH_2OH in rubber.

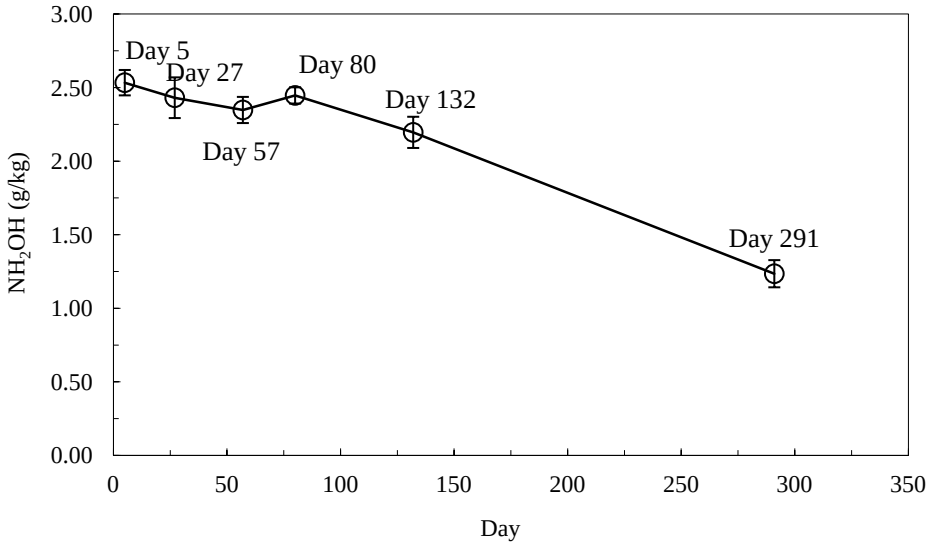

Figure K.1 The degradation process of NH_2OH measured in 3 g/kg RTEC rubber.

APPENDIX L
HOMOGENEOUS OF NH_2OH IN RTEC RUBBER SAMPLE

This experiment was performed for testing the homogeneity of NH_2OH in the rubber sample. Ten positions randomly collected from 3 g/kg RTEC sample were extracted using SLE method and then NH_2OH was analyzed by HPLC reversed-phase method. The result is shown in Table A.4 and Figure L.1.

Table A.4 Homogeneity check of rubber samples.

Sample	$(NH_2OH)_2.H_2SO_4$, Found, (g/kg)
1	1.09 ± 0.01
2	1.53 ± 0.06
3	1.48 ± 0.07
4	1.23 ± 0.09
5	1.32 ± 0.02
6	1.75 ± 0.08
7	1.34 ± 0.04
8	1.19 ± 0.06
9	1.17 ± 0.07
10	1.68 ± 0.04
Sample average	1.38
Within-samples SD	0.22
%RSD	15.9

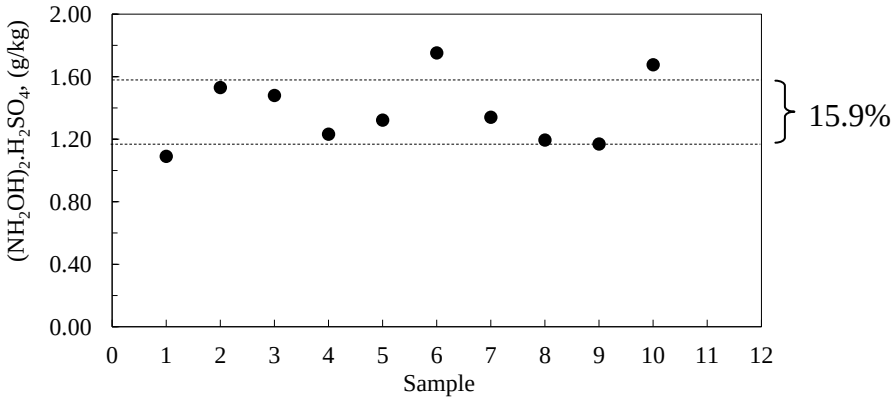

Figure L.1 Homogeneous of NH_2OH in RTEC rubber sample.

APPENDIX M
CALCULATION FOR DETECTION LIMIT AND QUANTITATION LIMIT

In this work, detection limit (DL) and quantitation limit (QL) was determined by following AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. 2013 written by Thompson and Lowthian (AOAC 2013).

DL is the lowest concentration of analyte that can be detected without reliable accuracy or precision. The calculation of DL is based on the variation of blank taken by:

$$DL = x_{Bl} + 3s_{Bl}$$

Where x_{Bl} is the blank value

s_{Bl} is the standard deviation of blank

QL is the lowest concentration of analyte that the signals can be quantitated with acceptable reliability. The QL is calculated by:

$$QL = x_{Bl} + 10s_{Bl}$$

In this part, we will show the calculation of DL from the analysis of NH_2OH standard using HPLC reversed-phase method. However, in this work, the signal of blank solution cannot be measured, therefore, $x_{Bl} = 0$ and s_{Bl} was calculated by using the concentration at the lowest detectable signal of NH_2OH instead. The calculation is shown below.

Table A.5 The results and the calculation for DL.

Peak area of 0.2 mg/L NH_2OH (n = 10)	**SD of signals**	**Linear equation**	**DL (mg/L)**	**Predicted QL (mg/L)**
969	136.7	y = 6166x - 545.61	0.15	0.31
665				
654				
926				
879				
685				
1025				
738				
901				
912				

QL will be confirmed once again by measuring NH_2OH at concentration of predicted QL: 0.31 mg/L (but, in the experiment, we tested with 0.4 mg/L of NH_2OH). 10 injections were performed. The results were calculated for mean, standard deviation and HORRAT.

Table A.6 The experiment for confirming QL.

Predicted QL (mg/L)	**No.**	**Found (mg/L)**
0.4	1	0.39
	2	0.37
	3	0.42
	4	0.34
	5	0.31
	6	0.37
	7	0.34
	8	0.39
	9	0.40
	10	0.38

Table A.7 The statistic calculation for confirming QL.

Mean	0.37
Standard deviation	0.03
Relative standard deviation, RSD_R	8.96
Predicted RSD, $PRSD_R$	18.2
HORRAT	0.491
%Recovery	92.7

$$\text{HORRAT} = \frac{RSD_R}{PRSD_R}$$

(The detail for HORRAT was shown in APPENDIX S)

$$\text{Recovery, \%} = \frac{(C_f - C_u)}{C_a} \times 100$$

Where C_f is the concentration of the fortified test samples

C_u is the concentration of the unfortified test samples

C_a is the calculated concentration of analyte added to the test sample

Table A.8 The acceptable recovery requirements from AOAC.

Concentration	**Recovery limits, %**
100%	98 – 101
10%	95 – 102
1%	92 – 105
0.1%	90 – 108
0.01%	85 – 110
10 µg/g (ppm)	80 – 115
1 µg/g	75 – 120
10 µg/kg (ppb)	70 - 125

The concentration of NH_2OH at 0.4 mg/L showed good precision because HORRAT was in range 0.3 to 1.3 and good accuracy because the recovery (92.7%) is not over than acceptable recovery limit of AOAC (75 to 120%). These informations confirmed that QL of this method was 0.4 mg/L.

APPENDIX N
THE HORWITZ EQUATION

In 1980, Horwitz, Kamps, and Boyer including the statistician Jung Keun Lee presented the coefficient of variation under reproducibility conditions (RSD_R) expressed as:

$$RSD_R=2^{(1-0.5\log C)}$$

In 1999, Michael Thompson transformed the equation to:

$$RSD_R, \% = 2C^{-0.15}$$

Where, C is the mass fraction of analyte

Table A.9 RSD_R calculated from different concentrations of analyte.

Concentration of analyte	**RSD_R**
10%	2.8%
1%	4.0%
0.1%	5.7%
0.01%	8.0%
1 µg/g (ppm)	16%
1 µg/kg (ppb)	45%
0.1 µg/kg	64%

Horwitz Ratio (HORRAT)

HORRAT is calculated from the ratio of RSD_R (the relative standard deviation culculated from experimental data) and $PRSD_R$ (the predicted relative standard deviation from the Horwitz equation), shown as:

$$\text{HORRAT} = \frac{RSD_R}{PRSD_R}$$

For repeatability conditions, the accetable values are between 0.3 and 1.3 and for reproducibility conditions, the acceptable values are between 0.5 and 2 (Rivera and Rodríguez).

For example, this table shown the results from intra-day precision that three concentrations of NH_2OH were tested with 10 replicated analysis. Mean, SD, RSD, HORRAT, and %recovery were calculated.

Table A.10 Example for the calculation of HORRAT.

No.	Found (mg/L)		
	0.5 mg/L (Exact)	**5 mg/L (Exact)**	**50 mg/L (Exact)**
1	0.57	4.93	50.97
2	0.57	4.99	47.61
3	0.63	4.95	45.42
4	0.59	4.84	46.07
5	0.61	4.95	47.40
6	0.57	4.92	49.53
7	0.59	4.99	49.10
8	0.65	4.89	49.33
9	0.60	4.90	50.86
10	0.63	4.95	48.76
Mean	0.60	4.93	48.5
SD	0.03	0.05	1.86
RSD_R	4.8	0.9	3.8
$PRSD_R$	17.6	12.5	8.8
HORRAT	0.3	0.1	0.4
%Recovery	120.2	98.6	97.0

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